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ENHANCING THE YIELD OF TARGET TISSUE AND SECONDARY METABOLITES IN *CALENDULA OFFICINALIS* L., A MEDICINAL PLANT

By

Christie Stewart

A Thesis submitted to the Faculty of Graduate Studies and Research
through the Department of Biological Sciences in
partial fulfillment of the requirements for the
Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada
2003
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ABSTRACT

Medicinal crops can be usefully studied in controlled hydroponic systems in which various factors can be manipulated to increase target plant tissue yield and secondary metabolite production.

In this project floral tissue and other plant organs of the medicinal plant *Calendula officinalis* and four important secondary metabolites: quercetin, rutin, isorhamnetin-3-O-glucoside and isorhamnetin-3-rutinoside were quantified under contrasting conditions in terms of phosphorus concentration, rate of nutrient supply and simulated foliar herbivory in a factorial experimental design. The objectives were to identify conditions that will maximize the yield of target plant tissue, maximize the production of secondary metabolites and minimize the variation in that value.

Phosphorus concentration was varied because this nutrient is important for plant growth, particularly during flower production. Increased phosphorus promotes inflorescence production, as well as increasing production of some secondary metabolites. Control of nutrient supply rate in hydroponic culture is critical for optimal plant growth. Some hydroponic techniques provide large volumes of solution with nutrient concentrations higher than soil solution concentrations, while others allow nutrient depletion by plants before complete solution replacement. This often results in deficiency symptoms and fluctuations in growth rate. Nutrient supply rates used in this study sought to minimize nutrient deficiencies and growth fluctuations. Selected plants in the study were also subjected to a clipping treatment, as numerous studies have shown that herbivory can induce increased growth ("overcompensation") and potentially to stimulate secondary metabolite production.

Yield of capitula was maximized under the intermediate phosphorus concentration (100 mg/L), and under the nutrient supply rate which provided plants with fresh nutrient solution less frequently. Foliar herbivory did not lead to overcompensation or increased flower production in *C. officinalis*. Production of target flavonoids per unit dry mass (g) of capitula was maximized under the high phosphorus concentration (200 mg/L), and under the nutrient supply rate that provided nutrients more frequently. Simulated foliar herbivory did not induce greater production of flavonoids. Maximum total mean yield of flavonoids/plant was seen at the intermediate phosphorus concentration (100 mg/L). Ungrazed plants produced greater flavonoid yield/plant than did "grazed" plants. The potential for more effective commercial production of these substances is discussed.

DEDICATION

I dedicate this thesis to my family and all of my friends for their immense amount of support, patience, faith and encouragement.

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GENERAL INTRODUCTION

Medicinal plant products: impurities and the need for quality control

It has been estimated that eighty percent of the world's population depends on traditional herbal medicines as their primary source of health care (World Congress on Medicinal and Aromatic Plants for Human Welfare (WOCMAP), 1992), and the North American market is growing rapidly (Greenwald, 1998). Between 1994 and 1998, sales in North America for natural dietary supplements, which include vitamins and herbal remedies, grew at greater than 10% a year (Greenwald, 1998), with an observed increase of 380% in the use of herbal remedies, between 1990 and 1997 (Eisenberg, 1998). There is a strong demand for increased quality, uniformity and safety standard in medicinal plant products. The present market demand is met by purchasing plant material from cultivated sources and using material which has been wildcrafted, or collected from natural populations, in jurisdictions where there is little legislation and/or regulation of the quality of the plants. In many cases there are no guidelines or requirements concerning particular plant constituents, or the quality of the growing environment (Rates, 2001).

As a result of the absence of legislation and/or regulation, many possible contaminants (including pesticides, heavy metals, undisclosed pharmaceutical additives, fungal toxins, and radiation), can be incorporated in the plant material. These can have toxic effects in consumers, which may counterweigh medicinal benefits (Januz, 1994; Chourasia, 1995; Ahmed, 2001; Ernst, 2002). In Canada, most raw materials of medicinal plant species are not cultivated in sufficient quantities and therefore have to be

imported or wildcrafted. North American imports of raw plant materials of medicinal plant species have increased by 40% in the last decade (Buzzanell, 1995); Canadian manufacturers are now importing 70% of all medicinal herbs, and, in one survey of their ingredients, 37% of these were found to be of low quality (Saskatchewan Agrivision Corporation Inc., 2001). In many countries, standards are not as stringent as those of Canada, regarding environmental pollution or product quality control (Ernst, 2001). Pollution from irrigation water, atmosphere, and soil plays an important role in contamination of medicinal plants by pesticides, heavy metals and occasionally radioactivity, especially in developing countries (Al-Saleh, 1994; Al-Kathiri, 1997; Mandal, 1998; Korobova, 1998; Duffy, 1999; Abou-Arab *et al.*, 1999; Rai, 2001). Often contaminants become dangerously concentrated, as plant materials are dehydrated and concentrated for product preparation (Rates, 2001).

Analysis of herbal plant material suggests chlorinated pesticides are quite common contaminants (Abou-Arab *et al.*, 1999; Zuin and Vilegas, 2000). High levels of organochlorides, including malathion, lindane, aldrin, dieldrin, DDT, clordane and endrin have been found in many species of medicinal plants, from Egypt, Portugal, Poland, Sri Lanka, China and elsewhere (Lutomski and Debska, 1974; Robin *et al.*, 1978; Zambo *et al.* 1989; De Silva and Thiemann, 1991; Abou-Arab *et al.* 1999; Zuin and Vilegas, 2000; Abou-Arab and Abou Donia, 2001). Zuin and Vilegas (2000) reviewed instances of pesticide residues in medicinal plants: twenty-eight studies reported evidence of organochlorine pesticides, while fifteen found evidence of organophosphorus pesticides; in both groups based on several hundred types of plant samples. Pesticide use in food plants is regulated in many countries, but legislation is lacking or largely ignored in many developing countries (Zuin and Vilegas, 2000). As a result of the persistence of

organochlorides in humans, they have been banned in some countries (e.g. DDT in the U.S. and Canada), yet are still used on crops quite frequently in other countries, which may then be imported to Canada for consumption. It is important to note that, in practice, large-scale field cultivation of medicinal plants is very challenging without pesticide use (Zuin and Vilegas, 2000).

Heavy metals are also a source of contamination of medicinal plants, due to a variety of sources, ranging from industrial and traffic emissions to the use of industrial waste and agricultural methods, such as cadmium-containing dung, organic mercury fungicides and the insecticide lead arsenate (Abou-Arab *et al.*, 1999). Officials in California screened 251 imported Chinese herbal products for heavy metals and undeclared (adulterated) pharmaceutical ingredients (Ko, 1998). Thirty-six products averaged 14.6 ppm of arsenic; 24 contained 10 ppm of lead; and 35 averaged 1046 ppm of mercury. Ayurvedic herbal remedies from India were analyzed for their mercury content and out of thirty -one tested, 30 exceeded the legal limit in India of 1ppm of mercury (Itankar, 2001). Sixteen of these exceeded the limit for mercury by more than two orders of magnitude. Health Canada (2000) suggests a safe level of mercury in sport fish at 0.5 ppm.

It has been shown that heavily-contaminated plant products play an important role in the accumulation in consumers of the overall body burden of toxic metals (Chizzola and Franz, 1996). For example, many reports describe lead, mercury, thallium, arsenic, and cadmium poisoning due to the use of Asian and Indian herbal medicines (Ernst, 1998, 2002). Heavy metals typically cause injury of the kidney, and symptoms of chronic toxicity such as abdominal pain, lethargy, diarrhea, and vomiting; renal failure and liver damage (Abou-Arab *et al.*, 1999), muscle wasting, seizures, harmful weight loss (Ernst,

2002), osteomalacia (softening of the bones), renal tumors and other carcinomas (Schumacher *et al.*, 1991), and psychological and neurological disorders (Rai *et al.*, 2001) have also been reported. In India, consumption of edible medicinal plants laden with arsenic contributes to overall high arsenic levels, detected in human urine, nail and hair samples, and in severe cases causing melanosis and squamous cell carcinoma (Mandal *et al.*, 1998).

Contamination of plants by radioactivity can occur through soil, water and atmospheric contamination. For example, radioactivity has accumulated in vegetation in Russia as a result of the Chernobyl accident (Korobova, 1998) and was found in six medicinal plant species tested in the Marshall Islands as a result of weapons testing (Duffy *et al.*, 1999).

Ernst (1998) reviewd incidents where the final products obtained from medicinal plants have been adulterated with pharmaceutical agents. This problem occurs as well as the other possible methods of herbal medicine contamination and may have dangerous detrimental effects on the consumer. Ernst (1998) also reviewed an investigation of Ayurvedic remedies, which revealed that several had been adulterated with undeclared conventional drugs, such as acetylsalicylic acid (Aspirin®) and acetaminophen (the active ingredient of Tylenol®). Ernst discussed a study of Chinese remedies shown to contain drugs, like mefenamic acid, used as an anti-inflammatory and analagesic, and diazepam, used as a sedative, muscle relaxant and anti-convulsant, both of which resulted in cases of massive intestinal bleeding. Ko (1998) reported that 7% of 251 Chinese herbal remedies investigated contained undeclared pharmaceuticals. Other pharmaceuticals that have been found in traditional Chinese remedies include non-steroidal anti-inflammatories, corticosteroids, and anticonvulsants, which have been responsible for health effects such

as agranulocytosis, Cushing's syndrome, and coma (Ernst, 2002). These "undeclared" drug adulterants present not only a serious problem in themselves, but also in their potential interaction with other drugs the individual may be taking. The risk to consumers should be minimized by implementing regulatory measures for quality control, or controlling the supply, either at the level of import or production.

An additional concern of possible plant misidentification occurs when source plants are obtained from natural, uncultivated sources through wildcrafting. Plant misidentification threatens the overall safety of consumers, and the quality of medicinal plant products. Different plants may be mistaken for the target plant, compromising human health and safety, especially in cases where the misidentified plant is considered toxic to humans. This may occur inadvertently by error, or deliberately, to save money or effort (Ernst, 1998). For example, in one incident contaminated raw plant material labeled as plantain (*Plantago major*) was imported, processed, and distributed in the United States. This plant material contained foxglove (*Digitalis lanata*) and ingestion of the products resulted in toxic levels of digoxin in patients (Slifman, 1998).

Another problem with the use of medicinal plants for pharmaceutical purposes involves the efficacy of the product being used. The effectiveness of a nutraceutical remedy depends on the quantity of the bioactive compound found in the product and the recommended dosage, as well as consumer-related parameters (age, sex, other drug use) (Capasso, 2000). Hepinstall *et al.* (1992) investigated commercially available products of Feverfew (*Tanacetum parthenium*) for parthenolide content, which is the bioactive metabolite. A wide range of parthenolide concentrations were found, ranging from <10μg (undectectable levels) to levels as high as 795μg/capsule. The recommended daily dosage for these products was one capsule/day, even in those products that contained

undetectable levels of parthenolide. Thus, depending on the brand, the consumer could ingest anywhere from 795µg/day to less than 10µg/day of parthenolide.

Presently, due to a lack of standardization of nutraceutical remedies, recommended dosages are at the discretion of the consumer or production company, and are based loosely on the amounts used in traditional medicinal systems rather than on controlled clinical efficacy trials (Elvin-Lewis, 2001). Inconsistencies also exist among tests for efficacy, which are also attributable to the quantity of the bioactive compound in the product or plant material being tested, as well as different methods and end-results used for testing efficacy. Most medicinal plant remedies in Western countries have never had rigorous scientific testing for efficacy (Capasso *et al.*, 2000). Standardization of recommended dosages, bioactive compound quantity/quality, and testing methods to determine the efficacy of the product, as well as thorough scientific testing and record keeping of all available medicinal plant products is needed if a persuasive case is to be made for the efficacy of medicinal plants for pharmaceutical purposes.

Legislation and good manufacturing practices

The North American market for medicinal plant products is worth more than twelve billion dollars annually (Sturdivant and Blakley, 1999). However, there is little scientific research carried out on methods of control of the quality and uniformity of these medicinal plant products, which remain in the food and nutritional supplement category rather than as a commercial pharmaceutical drug (Bast, 2002). Health Canada (2001) established the Natural Health Product Directorate in 1999 to support the development of a legislative framework for natural health products, in order to address issues such as safety, efficacy, licensing, labeling, and applications (Bast, 2002). Presently, in Canada,

under the Federal Food and Drug Act of 1994, if the product is to be sold as a drug with any health-related claims the regulations require Good Manufacturing Practices (GMPs) from the herbal manufacturer in order to be granted a Drug Identification Number (Sturdivant and Blakley, 1999). The alternative is to have the product sold as a "food" product, which forbids health claims or dosage recommendation on the label (Gillis, 1999).

When natural health products are to be distributed as a food product or supplement, the standards for manufacturing are voluntary and product review and approval is not required. In the United States, the Dietary Supplement Health and Education Act, which was established in 1994, regulates the marketing of dietary supplements that include natural health products under the general umbrella of "foods" (Bast, 2002). Under this Act, the manufacturer is responsible for the safety of the marketed natural health product and the proof of any representation or claim must be backed by "adequate evidence" to prove that they are not false or misleading (Bast, 2002). The company is not required to supply this information to the consumer or the Food and Drug Administration (FDA) unless it is a new dietary ingredient subsequent to the passing of the act in 1994. It is also the obligation of the manufacturer to establish its own manufacturing practice guidelines to ensure product safety (U.S. FDA, 2002). As a result, there is little consistency among safety practices for the production and sale of natural health products, especially with respect to the lack of standardized dosages, or the amount of each ingredient within a product. These decisions do not require FDA review or approval. Negotiations between the FDA and the medicinal plant industry are battling key issues regarding the establishment of GMPs, drug review procedure for over-the-counter natural products, and labeling rules (Sturdivant and Blakley, 1999). Under the U.

S. Nutrition Education Labeling Act of 1990, labels are required to state that the product is a supplement, to give a descriptive name of the product and the name and place of business, a complete list of supplemental ingredients and other ingredients, and any other contents that are not listed, such as the contents used to make the capsules (Sturdivant and Blakley, 1999; U.S. FDA, 2002). It is illegal for the labels to contain claims regarding the treatment, prevention or cure for any disease. Presently, manufacturers do not need to register with the FDA nor get approval for producing or selling natural health products (U.S. FDA, 2002).

Ecological concerns and a possible solution

Wildcrafting is the most common means by which medicinal plants are obtained (Ward, 1990). This poses concerns, including over-exploitation of natural populations, consequent genetic erosion and increased rates of extinction. Genetic erosion is the loss of genetic variability within a population due to decreased size and isolation of populations (Young *et al.*, 1996). As medicinal plants are wildcrafted without applying sustainable management, populations become smaller and possibly fragmented. As this occurs, there is an increased chance of inbreeding and a decrease of gene flow between populations, leading to a loss of heterozygosity and a shrinking gene pool, often producing a genetic bottleneck within the population (Young *et al.*, 1996). This effectively translates into a loss of fitness for individual plants and lower future viability for the population, increasing the extinction threat for the plant population.

Many highly-sought-after medicinal plants are now on the verge of extinction worldwide, and the list of threatened species is growing (Johnson, 2002). Presently in Canada, thirteen medicinal plant species are considered Endangered, twelve as

Threatened, and another ten are in the Special Concern category, from a total of 35 medicinal plants that have been assessed by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC, 2002) (Table. 1.1). Two examples of interest because of their popularity and human benefit are American Ginseng (*Panax quinquefolius*) and Pacific Yew (*Taxus brevifolia*). American Ginseng has been intensely harvested from natural populations (Lantz, 2001); this is believed to be an unsustainable level when compounded with threats to its natural habitat. This species is protected internationally by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1975. The provincial governments in both Ontario and Quebec have made it illegal to collect and to export wild American Ginseng (Robbins, 1998).

In the case of Pacific Yew, which supplies the important anti-cancer drug taxol (Taxol®) (Jennewein and Croteau, 2001), it, too, has been intensely harvested since the early 1970s and its harvest is presently regulated in British Columbia (where it is distributed in Canada) under the B.C. Ministry of Forests (Lantz, 2001). Isolation of 2.5 kg of taxol requires approximately 2.5×10^7 kg of *T. brevifolia* bark, equivalent to 12,000 trees (Rates, 2001). As a result, natural populations have become imperiled, which in turn threatens the natural supply of this drug (Rates, 2001). Most taxol is produced today by semi-synthesis via isolation of a taxol intermediate from the needles of *Taxus baccata* or *T. yunnanensis*, since complete artificial synthesis is very difficult and not economically efficient (Jennewein and Croteau, 2001). Both of these cases exemplify the need for alternative methods of production not only for highly sought after, economically important medicinal plants, but all medicinal plants with market potential.

Cultivation of medicinal plants is frequently suggested as an option to reverse the depletion of wild stocks (e.g., Labadie 1986; Lawrence, 1993), and this would also reduce

problems of contamination and misidentification. However some medicinal plants may not be readily adapted for field cultivation. The agroecosystem of the cultivated field may lack the physical and chemical variables necessary for the plant to grow (Craker, 1999). Plants that are physiologically or genetically adapted to particular types of environmental conditions might not grow well or survive if they were cultivated in conditions they were not adapted to. By the same argument, not all medicinal plants would be readily adaptable to all traditional types of hydroponic systems, especially plants which do not require large amounts of nutrients or water. This was well illustrated by Dorais *et al.* (2001), who evaluated the potential growth of medicinal plants in a floating raft growing system. Seven of the nine medicinal species studied proved well-suited to this type of hydroponic system. Herbaceous species are more likely to be grown using hydroponic cultivation than woody species, like trees. Unlike field cultivation, in greenhouse hydroponics, environmental conditions, as well as the hydroponic technique used, can be adjusted to match the natural conditions the species are found in.

Hydroponic cultivation allows production under controlled climate conditions, with a control of contaminants and pesticides, and proper plant identification, all resulting in greater product quality and uniformity, which is not found with field cultivated or wild populations. Natural plant populations are genetically variable. This leads to variation in the concentrations of the secondary metabolites (Kaundun, 1998; Jennewein and Croteau, 2001). The environment is also variable and heterogeneous with respect to nutrients and climatic conditions, which can also have a significant effect on the make-up as well as the concentrations of secondary metabolites (Kaundun, 1998). The use of cultivated plants allows the production of more homogeneous crops, ensuring greater uniformity of bioactive compounds, compared with wildcrafted materials.

In principle, since hydroponic cultivation allows for contaminant- and pesticide-free conditions, with the added value of homogeneous crops producing uniform concentrations of secondary metabolites, they may be considered of higher quality than field crops of wildcrafted plants (Dorais *et al.*, 2001). Medicinal plants and plant products deemed to be of high quality have greater market value, especially those that are certified as organic (Sturdivant and Blakley, 1999). This suggests that a grower producing high quality plants with a higher market value will have a higher net income than if the plants were of lower quality. At the same time, greenhouse hydroponic cultivation can generate much higher yields than field-grown crops, with up to three growing cycles per year, adding to the production income (Mirza, 1996; Papdopoulos, 2000).

Introduction of medicinal plant cultivation would also fulfill the need to diversify greenhouse crop options, and allows for potential commercial cultivation. Presently, commercial greenhouse hydroponic crops include of tomatoes, cucumbers, lettuce and peppers (Papadopoulos, 1994). Most of the market is saturated, with little room for new growers to enter the market competitively (Coatney, 2001). Recently, the U.S. hothouse tomato growers (EuroFresh, Sunblest Management LLC, HydroAge, Village Farms, Sunblest Frams LLC, and Carolina Hydrponic Growers Inc.) filed an "anti-dumping" petition with the U.S. International Trade Commission against Canadian growers, whom they accused of "dumping" hydroponically grown tomatoes, because hothouse tomatoes were being imported and sold well below U.S. growers prices, which is an unfair trading practice (Coatney, 2001). When the Canadian tomatoes entered the American tomato market, it became, these groups claimed, unstable with resultant financial losses for American growers (Coatney, 2001). The cultivation of new medicinal plants by

greenhouse hydroponics gives growers other options for putting a competitive new product on the market.

Plant nutrient availability

Medicinal plant cultivation in greenhouse hydroponics can be used to supplement the growing market for natural health products. The implementation of greenhouse hydroponics requires extensive knowledge of plant physiology, in particular in the context of a plant's response to available resources, when generating optimal yields and minimizing financial loss. One major component of these resources involves plant nutrient concentration and availability.

All plants require certain basic nutrients for growth and development. Phosphorus is a critical macronutrient for plants (Raghothama, 2000), used in the production of nucleic acids and phospholipids, in energy metabolism, in the activation of metabolic intermediates, and the regulation of enzymes (Rausch and Bucher, 2002). It is abundant in fruits and seeds, and also in the root hairs (Papadopoulos, 1994). It is required at variable rates during the growth cycle (Papadopoulos, 1994), such that there are changes in the relative demands for different nutrient elements in an optimally allocating plant as it grows (Raven, 2001). Phosphorus is a critical component of energy carrier molecules, such as ATP, GTP and ADP; it is also used, for example, in the biosynthesis of fats.

It is widely found that increasing phosphorus, as a fertilizer, will promote reproductive yields (Egle *et al.*, 1999) and inflorescence production, particularly when phosphorus is otherwise limiting in the system; as well as increasing some secondary metabolite production, such as tannins and phenolics (Feller, 1995). Increasing the phosphorus supply in plants has also been shown to alter the salt tolerance of many crops

(Zaiter and Saade, 1993). Plants absorb phosphorus in its inorganic form (Pi) (usually as ionic orthophosphate, PO_4^-) and it exists in several different forms in solution (Rausch and Bucher, 2002). The availability and concentration of each form is dependent on soil pH (Kawai, 1980). Uptake of Pi is greatest between pH 4.5-6.0, in the $H_2PO_4^-$ form (Rausch and Bucher, 2002) (Fig. 1.1). In phosphorus-deficient soil, phosphorus uptake can be increased due to the coupled release of H+ ions with P uptake, which decreases soil pH and increases availability of $H_2PO_4^-$ (Tang *et al.*, 2001). Excessive phosphorus levels may induce iron and zinc deficiencies through ionic competition for uptake (Chaney and Coulombe, 1982; H. A. Mills, 2001, pers. comm.), and a high concentration of phosphorus (at 240 ppm of P) has been found to be toxic to tomato plants, inducing interveinal chlorosis (H. A. Mills, 2001, pers. comm.).

Phosphorus is often limiting in natural ecosystems, due to soil-associated factors (Raghothama, 2000). The prevalent form of phosphorus in the environment is the oxidized anion, phosphate PO_4^{3-}(Rausch and Bucher, 2002). Available phosphorus (PO_4^-), which represents the preferred form for assimilation in plants, is less than total phosphorus present in soils due to its interaction with cations (Rausch and Bucher, 2002) and its conversion into organic complexes by microbes (Gyaneshwar *et al.*, 2002). Pi (PO_4^-) is absorbed into soil particles, clay minerals and surfaces of calcium and magnesium carbonates or forms insoluble precipitates with cations, like iron, aluminum, and calcium, which makes it less available for uptake (Rausch and Bucher, 2002).

Adaptation to low nutrient environments

Different species require different amounts of phosphorus for optimal growth, which suggests that a response to its deficiency is one aspect of the physiological

adaptation of populations to local soil conditions (Christie and Moorby, 1975). Those species that require low phosphorus for optimal growth appear to have adapted to phosphorus-limited environments (Christie and Moorby, 1975), which are typically the alkaline calcareous soils (calcisols) that represent more than 25% of the earth's surface (Ragothama, 2000). These soils are found mostly in arid or semi-arid or Mediterranean climatic conditions, often containing sparse natural vegetation of xerophytic shrubs and ephemeral grasses (Henkin, 1996). The limitation of phosphorus in these soil conditions is due to the formation of unavailable calcium phosphates as apatite, which results in a high pH, and is also typically limited in water, nitrogen, zinc and iron, along with nutritional imbalances between potassium and magnesium with calcium (Carreira, 1997). Vertisols are also P-limited, due to P-fixation to the high concentrations of calcium and magnesium minerals found in these soils, and the soil is N-limited and has a high clay content as well (FitzPatrick, 1980). They are mostly found in semi-arid tropics that have alternating wet and dry seasons (FitzPatrick, 1980). Other P-limited soils are acidic soils, called acrisols and ferralsols, found mostly in tropical and subtropical landscapes under high rainfall conditions containing the world's tropical rainforests, and with soils having toxic levels of aluminum and P limitation due to a high capacity for P fixation (FitzPatrick, 1980). Podzols are also P-limited acidic soils occurring mostly in the cool temperate and boreal regions and to a lesser degree in many parts of the tropics, with toxic levels of aluminum and nitrogen restriction (FitzPatrick, 1980).

Species which grow in nutrient-poor soils have usually evolved low requirements for certain nutrient elements (Clarkson, 1967) and/or evolved higher nutrient use efficiency for the limiting nutrient (Lajtha and Klein, 1988). A low nutrient requirement for phosphorus and/or high nutrient-use-efficiency of phosphorus reduces the dependence

of plant growth on high levels of soil phosphorus. Adaptation to low nutrient availability by low requirements for that nutrient suggests an adjustment in the uptake and metabolism of the element in question. Unfortunately this is difficult to verify (e.g. Clarkson, 1967). Higher nutrient use efficiency as an adaptive response to limiting nutrients, on the other hand, can be experimentally demonstrated (e.g., Lajtha and Klein, 1988). Lajtha and Klein investigated the effect of varying nitrogen and phosphorus availability on nutrient-use efficiency in a desert evergreen shrub, *Larrea tridentata*, which grows in nutrient-, as well as water-limited soils. They observed that N and P use efficiency decreased with increasing N and P availability, respectively (achieved by increasing their concentrations). Nutrient use efficiency increased for both N and P when the availability of the other nutrient increased. Their results support higher nutrient use efficiency occurring as a response to limiting a nutrient. In addition, studies by Christie and Moorby (1975) and Clarkson (1967) indicated that when the nutrient becomes available for species growing in phosphorus limitation, a decrease in nutrient use efficiency occurs, illustrated by higher tissue concentrations of phosphorus. These increased tissue concentrations in slow-growing species appear to have luxury consumption of the limiting nutrient during times of nutrient flushes (Clarkson, 1967; Christie and Moorby, 1975).

An alternative adaptive response to limited nutrient availability was suggested by Gerloff (1963), that plants growing in nutrient-poor soils, such as phosphorus-limited soils, may have adapted to these conditions by having a slow growth rate and low yield (dry mass). Christie and Moorby (1975) examined this hypothesis by investigating the physiological basis for species differences in yield, and nutrient responses to varying supplies of phosphorus concentrations. They compared the growth and yield responses of

two native semiarid grass species from Queensland, Australia; Mitchell grass (*Astrebla elymoides*) and mulga grass (*Thyriodolepis mitchelliana*), and an introduced pasture sown grass, Biloela buffel grass (*Cenchrus ciliaris*). All three were experimentally grown in solution culture. Both native communities of the native grasses are naturally low in soil phosphorus concentration; with the mulga grass community being lower than the Mitchell grass, and the introduced buffel grass occurring naturally in communities with much higher soil phosphorus concentration. Buffel grass showed an increase in yield in response to increasing phosphorus, as did the Mitchell grass, but to a lesser extent than the mulga grass. Mulga grass also showed a lower requirement for external phosphorus concentration for optimal growth than did Mitchell and buffel grass. Lastly, the native grass species found growing in phosphorus-limited soils had much lower relative growth rates than the introduced species. It seems like that they have adapted these characteristics because plant growth rate and reproduction is dependent on nutrient availability. Nutrient-poor soils may favour those plants that can adapt/acclimate to environments with limited nutrient availability, whereas plants dependent on high nutrient delivery will fail to grow optimally or survive as they drop below the compensation point. This supports the idea of adaptation to nutrient-poor soils by low nutrient requirement, since in nutrient poor soils the required nutrients for growth are deficient or minimal, and by adapting a slow growth rate and low reproduction allows the plants to survive and reproduce in these conditions (Gerloff, 1963).

Clarkson (1967) studied physiological responses to phosphorus levels in soil and in hydroponic culture, in three *Agrostis* species; *A. setacea*, *A. stolonifera*, and *A. canina*. *A. setacea* most often grows in characteristically sandy podzols with a low pH and low phosphorus availability. *A. canina* has been shown to have wide edaphic tolerances while

A. stolonifera grows in soils that have more than four times as much available phosphorus than Bicton soils. When all three species were grown in Bicton soils, the two species, *A. stolonifera* and *A. canina*, that are not found naturally in phosphorus-limited environments failed to establish seedlings due to the low pH, while *A. setacea* maintained a slow constant growth rate. When the three species were grown in solution culture with phosphorus supplied at a high level to minimize depletion effects within the solution, *A. setacea* again showed a lower rate of growth than *A. stolonifera* and *A. canina*. In his review Chapin (1980), cited ten papers that reported an inherent low rate of growth for species growing in nutrient-limited habitats.

Low growth rates have also been shown to be a characteristic of *Eucalyptus* spp. in Australian soils having low phosphorus availability (Beadle, 1962). This low rate of growth is closely correlated with the essential need for phosphorus in protein synthesis (Loveless, 1961) such that if phosphorus is available in quantities that are less than what is needed to synthesize protein-based tissues, then the plant will reduce its rate of growth, or possibly prevent further growth until phosphorus becomes available. Protein synthesis has also been correlated with the availability of nitrogen (Loveless, 1961) as well as the proportional relationship between phosphorus and nitrogen uptake (De Magalhaes *et al.*, 1998).

Plant species in each of the previously-mentioned phosphorus-limited habitats have a commonality among them besides environmental nutrient limitation. The habitats also contain species with high levels of secondary metabolites; as a result they are often used for medicinal or culinary purposes. The phosphorus-limited Acrisols and Ferralsols typical of tropical rainforests contain a high diversity of species (Janzen, 1974), many of which have both phenolics (Coley, 1983) and high alkaline-based metabolites, called

alkaloids (Levin, 1976). Many species in the calcareous soils of Mediterranean-type climate have been noted for their aromatic nature and high oil content in species such as sage, rosemary, thyme and oregano that are characteristically evergreen with small, leathery leaves and thick cuticles (Woodward, 1997). This thickening of cuticles and leathery appearance is known as sclerophylly or the hardening of leaf tissue (Beadle, 1968). Other succulent perennial forbs from this climate type include *Eucalyptus* and *Aloe* species (Woodward, 1997). The humic-rich podzols of tropical, as well as northern boreal forests are dominated by plant families, especially conifers, containing high levels of phenolic compounds and terpenoids (Janzen, 1974; McNaughton, 1983), which are carbon-based secondary metabolites (Chrispeels and Sadava, 1996). Most of these species are also sclerophyllous in nature, with leaves having a leathery texture, high in lignins and waxes (Janzen, 1974), a high-fibre content and well-developed cuticles (McNaughton, 1983).

Many medicinal plants are native to xerophytic habitats that are often low to deficient in soil phosphorus, as well as other nutrients (Beadle, 1968) and water (Yaalon, 1997). Mediterranean climates are characterized by xerophytic habitats which have winter rains (three times that of summer rains) and warm dry summer months with moisture deficits (Yaalon, 1997). This is known as a xeric moisture regime. The vegetation type adapted to this climate is termed a xerophyte (Beadle, 1962). This xeric moisture regime of Mediterranean climate exists on all continents, typically on the western parts between the cooler temperate zone and hot dry desert zone (Yaalon, 1997), although it is also found on the north-west coast of Africa and south-south-west coast of Australia. Along with being P- and N-deficient, the soils of this region are also very calcareous and alkaline (Henkin *et al.*, 1996; Cocks and Osman, 1996; Carreira *et al.*

1997; Yaalon, 1997). A key characteristic of plant growth in limited phosphorus is sclerophylly, which is typical of, but not limited to, xerophytic species (Loveless, 1961). Sclerophylly is the hardening of leaf tissue which is thought to be an adaptive response to dry habitat to protect the leaves from water loss (Beadle, 1968), but has also been recognized as a possible response to limited nutrients, specifically phosphorus (Loveless, 1961).

Sclerophylly results from the thickening of the cuticle and outer epidermal wall and abundant sclerification, especially of the vascular bundle sheaths and leaf margins (Turner, 1994). Loveless (1961) developed an index for quantifying sclerophylly, based on the ratio of crude fibre to crude protein concentration of the leaf, with sclerophylls having a higher fibre content (higher ratio) than mesophylls. This trait is also suggested to be an adaptive defense mechanism against herbivory, such that plants with scleromorphic leaves are less likely to be grazed due to the difficulty of grazing hard leaf tissue (Janzen, 1974). Coley (1983) found the occurrence of sclerophylly to be the best predictor of where high rates of herbivory will occur in tree species in tropical lowland rainforest. It may also act as protection against a harsh environment for the long-lived tissues of slow-growing species in nutrient limited habitats (Turner, 1994).

Nutrient supply rates

In the past, limitations between the roots and their environment were removed by growing hydroponic crops under luxurious water and nutrient supply (Papadopoulos, 1998) that are higher than found in most naturally occurring environments (FAO, 2000). Hydroponic techniques that allow nutrient depletion by plants before complete solution replacement often result in deficiency symptoms and fluctuations in growth rate (Stadt *et*

al., 1991). The deficiency symptoms and growth fluctuations are usually due to imbalances in nutrients, nutrient ratios, pH and electrical conductivity (EC) caused by unequal uptake of some nutrients over others, or ionic competition. The EC in hydroponic solutions reflects the concentration of ions present and available to the plant for uptake, which regulates the osmotic potential of nutrient solution (Papdopoulos, 1998).

If the water supply contains nutrients in excess, greater than what is required for optimal plant growth, a buildup of certain nutrients in plant tissues, especially leaves, by way of luxury consumption, appears an inevitable response (Papadopoulos, 1994). If ions are in excess in nutrient solutions, resulting in a high EC value, the roots will not uptake some nutrients due to osmotic stress which reduces water potential at the root hair surface, causing a reduction in growth (Kramer, 1969). The plant will continue to uptake water and transpire thereby continuing to increase the ionic concentration in nutrient solutions. The alternative response of the plant to balance osmotic stress is to lose water through the root surface, resulting in lost turgor pressure in the cells. Ionic interactions at the root hair surface also occur by competitive inhibition of ions for the same carrier site (Chapin, 1980).

The above pair of examples illustrate that increasing the concentration of nutrients is not always beneficial, as traditionally thought, but in fact may be detrimental to plant growth, since careless nutrient application can lead to nutrient imbalances (as well as being costly for growers, and wasteful, leading to environmental pollution, Papadopoulos, 1998). Control of nutrient supply in hydroponic culture is critical for optimal plant growth, as measured by plant growth rate, reproduction or fitness, but deep culture solution and open run-through trough techniques that supply large volumes of nutrients

and water leads to overload, rather than control of plant nutrients or growth. Supply rates of nutrient solution provided in hydroponic systems for tomatoes grown in rockwool culture (fall crop) range from 0.4L/plant/day to 1.0L/plant/day over the course of plant growth (Papadopoulos, 1998). In the same conditions for commercial cucumbers the nutrient supply rate ranges from 0.8L/plant/day to 3L/plant/day (Papadopoulos, 1994).

Although the overall nutrient concentrations are important in maximizing plant growth, especially nutrients that are the limiting factor for growth, the frequency of nutrient supply has been shown to be quantitatively more important in solution culture (Lapointe, 1985). Specifically, Lapointe (1985) found this had the greatest impact on the seaweed *Gracilaria tikvahiae* that naturally grows in a P-limited environment, whereby the rate of uptake of PO_4^{3-} increased with decreasing the frequency of nutrient supply (i.e. increasing nutrient limitation). This suggested an enhanced ability for uptake of PO_4^{3-} under P limitation (Lapointe, 1985). The growing environment of *Gracilaria tikvahiae* is also N limited (Lapointe, 1985). In the same study, Lapointe (1985) also looked at the effect of the frequency of nutrient supply with respect to the uptake of N while both P and N were limited. The uptake of NH_4^+ was not affected by the change in supply frequency as was P uptake when the rate of frequency was decreased, suggesting that the uptake of P is more greatly affected by the rate of nutrient supply than the uptake of N in a nutrient-limited environment. The rate of supply of P is more critical to plant growth than the rate of supply of N in environments that are both P- and N-limited.

Herbivory: its implication in overcompensation and secondary metabolite production.

"Overcompensation" following herbivory is an increase of seed yield or vegetative productivity following natural or simulated gazing, in comparison to plants

that are left ungrazed (Paige and Whitham, 1987). There are several hypotheses about the mechanisms that lead to overcompensation in plants due to herbivory. The first is growth overcompensation results from the reallocation of assimilates from storage organs such as roots (McNaughton, 1983) where the root biomass decreases most likely as a result of the carbohydrate reserves in roots is being reallocated to support rapid above ground vegetative growth (Li *et al.*, 1997, Becker *et al.*, 1997; Rodriguez and Brown, 1998). A second possible mechanism for growth overcompensation relies on the hormones present in invertebrate saliva to functionally trigger or promote plant growth, as well as secondary metabolite production (Walling, 2000). Dyer and Bokhari (1976) found an increase in the number of tillers in hydroponically grown grass species (*Bouteloua gracilis*), following grazing by grasshoppers (*Melanoplus sanguinipes*), a much higher number of tillers than plants that had been mechanically clipped, suggesting a salivary stimulus which affected the subsequent regrowth of plants.

A third mechanism by which overcompensation may occur is by the release of dormant meristems and buds from herbivory of dominant apical meristems (McNaughton, 1983). This hypothesis is supported by results of studies showing an induction of vigorous regrowth from basal meristems after the removal of apical dominance, greater than plants that were not grazed (Huhuta, et al., 2000). These three mechanisms may not be mutually exclusive, but each may be an evolutionary and ecological response to herbivory.

Paige and Whitham (1987) addressed supporting evidence for the first two hypotheses in their study investigating overcompensation in response to ungulate grazing and simulated herbivory on scarlet gilia (*Ipomopsis aggregata*). They found that in this species overcompensation does not result from the reallocation of biomass from roots. In

fact, they found the opposite for scarlet gilia, in that both the natural and simulated grazing resulted in an increase of root biomass compared to the ungrazed controls. Paige and Whitham (1987) also found that, although the natural and simulated-grazing plants produced significantly more inflorescences and fruits than the control plants, the natural grazed and simulated grazed groups did not differ from each other in these measures of fitness. Hence, in scarlet gilia, it does not appear that hormone transfer in mammalian herbivores induces the overcompensation in the number of inflorescences and fruits. Nonetheless, scarlet gilia appears to benefit from being eaten by some mechanism of overcompensation which is not fully understood, nor is it clear why plants would have increased fitness due to an overcompensatory response evolved from exposure to herbivores.

Numerous other studies have shown that herbivory can induce increased growth due to the overcompensation response (e.g., McNaughton, 1983; Paige and Whitham, 1987; Nilsson *et al.*, 1996; Papatheodorou *et al.*, 1998; Agrawal, 2000) and stimulate secondary metabolite production (Collantes *et al.*, 1996; Litvak and Monson, 1998). Secondary metabolites are often produced by the plant in response to environmental stress and/or as a defense against herbivores or pathogens. It is thought that plants under greater stress (decreased nutrient availability/ herbivore attack) would therefore produce greater amounts of secondary metabolites (McNaughton, 1983). Collantes *et al.* (1997) and Gianoli and Niemeyer (1998) both found an induced increase in the concentration of hydroxamic acids, which are a class of secondary metabolites that play a role in herbivore resistance, in experiments using rye (*Secale cereale*), which was exposed to simulated defoliation, and wild wheat (*Triticum uniaristatum*), which was grazed by aphids. Likewise, Litvak and Monson (1998) observed a significant increase in the production of

monoterpenes in several conifer species which were treated with simulated herbivory, and an even greater response in ponderosa pine (*Pinus ponderosa*) experiencing natural herbivory by tiger moth larvae (*Halisdota ingens*). These increases in monoterpenes occurred despite the fact that they are also constitutively present in the tree species studied. It has also been found that grazed plants may overcompensate, such that the plants have greater fitness when they are damaged compared to plants that are undamaged through increased vegetative or reproductive growth or output; however this is most likely to occur under optimal environmental conditions (Maschinski and Whitham, 1989; Agrawal, 2000). Plants that grow in soils low in nutrients cannot acquire sufficient resources to support rapid regrowth after herbivory; these slow-growing plants are therefore less likely to show overcompensation (McNaughton, 1983).

A plant's response to herbivory is not consistent among taxonomically-similar species, and varies with the intensity and timing of herbivory, and the environmental conditions at the time of herbivore action (Lowenberg, 1994; Escarre´*et al.*, 1996; Pilson, 2000). For example, Escarre´ *et al.* found that the reproductive biomass of members of the family Asteraceae are more greatly affected (increasing for the plant species *Picris hieracioides*) by herbivory occurring later in growth, such that there is greater overcompensation in reproductive tissues, than in vegetative biomass. In contrast, early herbivory, especially prior to the formation of reproductive structures, can reduce the biomass of reproductive organs (Rodriguez and Brown, 1998) in addition to reducing the total above-ground biomass (Papatheodorou *et al.*, 1998). Since, herbivory can be found to increase the formation of reproductive structures, at a specific growth stage and

intensity, it can be used beneficially in a cultivation practices as a manipulation method for increasing yields, as well as increasing the production of secondary metabolites.

Research objectives

Use of commercial cultivation could eliminate concerns in producing high-quality plant products, and aid in the supply of material from highly sought-after plants. Although greenhouse plants grown in soil-based medium eliminate most potential concerns, such as a pesticide-free environment, soilless media, like hydroponics, provide an opportunity for better nutritional control and savings on water and fertilizer that cannot be provided by soil-based media (Papadopoulos, 1994, 1998; Dorais *et al.*, 2001). Soil-based media require greater amounts of nutrients and water due to leaching (Papadopoulos, 1998) as well as the fixation of some nutrients to soil particles (Papadopoulos, 1994). In addition, for optimal plant growth the soil would need to be comparatively similar to the soil conditions to which the plant is adapted. Greenhouse hydroponic cultivation of medicinal plants better addresses the concerns facing this potentially lucrative industry. This method will not only ensure high quality plant products, but also offers alternative crops, effectively diversifying the currently vulnerable greenhouse market (and provide an alternative to over-exploitation of wild populations) (Agriculture and Agri-food Canada, 1998). With hydroponic greenhouse cultivation, contaminant exposure can be minimized, while yield of commercially important plant tissues and their medicinally active constituents can be maximized by optimizing growing conditions. In this study the variables investigated were phosphorus concentrations, the rate of nutrient supply, and level of simulated herbivory performed as a clipping treatment, with attention to their effects on growth, yield and secondary

metabolite production in *C. officinalis*. The target tissue is the flower of the medicinal plant *C. officinalis*, and the target secondary metabolites are a group of flavonoids that have been identified within the flower in other studies.

Calendula officinalis

The medicinal plant *Calendula officinalis* (Asteraceae), the common pot marigold, which is the focus of the present study, is an annual species native to the Mediterranean region, which has been used for centuries medicinally (Duval, 1993). Habitats in the Mediterranean are typically xeric during the summer months (Yaalon, 1997). The Mediterranean-type climate is island-like in character in that it occurs in isolated sections of continents between 30° and 40° latitude on the west coasts of continents where there are cold-offshore currents (Yaalon, 1997). There is a high degree of endemism, especially in the flora, in which many of the shrub flora are aromatic and contain highly flammable oils (Woodward, 1997). Included in the Mediterranean climate-type are the fynbos of South Africa and the serpentine soils of California that contain some of the most unique and highest diversity of vegetation known (Woodward, 1997). Plants adapted to this region also typically show high production of antiherbivore defenses (Vogiatzakis and Griffiths, 1999), and it is these compounds that are often the bioactive secondary metabolites of interest in pharmacology (Briskin, 2000). *Calendula officinalis* has characteristics typical of a xerophytic species, including a thick leaf cuticle with waxy resins, and hairy leaves (pers. obsv.), and as a native species of the xerophytic habitat within the Mediterranean (Duval, 1993) it has adapted to an environment of limited water and nutrient availability (Yaalon, 1997).

The Asteraceae is most diverse in arid and semi-arid climates (Zomlefer, 1994). *Calendula* is one of two representative genera of the Calenduleae tribe, which contains 7 genera and 110 species, with its distribution confined mainly to Africa and the Mediterranean region (Zomlefer, 1994). The Latin name *Calendula* refers to the fact that this annual plant is in continuous bloom for many months (Jost, 1983). This makes the species in this genus a practical and attractive as greenhouse crops, in that there can be a continual year-round harvest. *Calendula officinalis* is a hardy species that prefers full sun or partial shade for optimal growth. Its hardiness is illustrated by the fact that flower production is little-influenced by wide-ranging climatic conditions, such as temperatures ranging from 2.8-26.8°C and low rainfall (12.7- 87.6 mm/growing period) (Piccaglia *et al.*, 1997).

In soil field crops, the seeds germinate in 7 to 14 days at a pH ranging from 5-8 (Sturdivant and Blakley, 1999). The plants grow to 60 cm in height with a radial spread of 30- 60 cm (leaves range from 15- 30 cm in length). *Calendula officinalis* produces pale green, simple, rosette leaves in which the leaf blade completely encloses the petiole (leaf mid-rib) (Figure 1.2). The leaf is thick and fleshy, exuding a very aromatic sticky, resin substance (calendulin) that adheres to the leaf (Jost, 1983). *Calendula officinalis* is a long-day qualitative plant, with an exceptionally low photoperiod requirement of 6 hours and 30 minutes in a 24 hour cycle for inflorescence initiation, although it appears to be insensitive to photoperiod until the four-leaf stage (Sawhney *et al.*, 1981). Bolting occurs after approximately six weeks of rosette growth and stems terminate in pointed terminal buds, surrounded by bracts. The buds and capitula show regular, radial symmetry. Capitula are apparent anywhere from 57 days after planting in soil beds in a

greenhouse (Han and Yeam, 1978), to 80 days after direct sowing in a field crop (Sturdivant and Blakley, 1999).

The 'heads' or capitula (composite inflorescences characteristic of the Asteraceae) appear as flat discs surrounded by two or three circles of elongated ray florets. The pistillate ray florets in *C. officinalis* have been shown to have greatest total content of flavonoids (Masterová *et al.*, 1991). The petals on these florets range from dark orange to pale yellow or a combination of these colours, and can change colour while in bloom. A single plant may also bear differently coloured capitula. As in the related species, sunflower (*Helianthus annuus*), the capitula are photonastically sensitive, in that they face open towards the sun and follow its path from sunrise to sunset, when the capitula close with the diurnal cycle of day and night on a 12 hour rhythm (Harder *et al.*, 1967). Flowering continues over a period of 6 to 8 weeks, sometimes as long as 12 weeks (Sturdivant and Blakley, 1999). In intensive field cultivation systems it has been shown that after the first removal of the flower heads the second round of flower harvesting is always more productive (Piccaglia *et al.*, 1997). This suggests that continuous floral removal may promote capitulum production, thereby increasing the cumulative target tissue yield per plant. If capitula are left intact, and the harvest is allowed to go to seed, the overall crop output of capitula is significantly reduced (Sturdivant and Blakley, 1999).

The capitula have the greatest fresh and dry weights when they are fully developed (Kasprzyk, 1970) and the highest amount of triterpenoids (free, ester, and glycoside form) and certain sterols (β-amyrin, erythrodiol, and oleanolic acid), although harvest date of the mature capitula and climate appear not to affect flavonoid and carotenoid content (Picagglia *et al.*, 1997).

Seeds of *C. officinalis* are polymorphic, with three seed types: cymbiform seeds (bird-claw-like), vermiform seeds (half-moon shaped), and rostrate seeds (having tiny wart-like bumps, horns and bristles) (Jost, 1983). Only the seeds of the innermost seed ring, closest to the outer edge of the central disc of the flower, contain viable embryos (Jost, 1983). The seeds have an oil content of 14-20%, with calendic acid accounting for 55-61% of the oil fraction (Breemhaar and Bouman, 1995). Based on this high oil fraction, *C. officinalis* has been proposed as a new oilseed crop for agriculture (Angelini *et al.*, 1997) with the potential to replace certain petrochemical building blocks with renewable resources because of their unusual fatty acids, such as calendic acid. They also show the potential to expand the existing range of oleochemical base materials (Muuse *et al.*, 1992). Synthetic derivatives could, for example, be used as binders in paints and coatings (Fritsche *et al.*, 1999). Unfortunately, from the oil-seed point of view, the indeterminate flowering pattern of *C. officinalis* produces an undesirably long maturation period, which would delay seed harvest (Breemhaar and Bouman, 1995). It is considered a heliophilic plant, in that increased irradiance will increase total dry mass production (Wassink and Van Den Noort, 1981), but an increase in photoperiod above 12 hours promotes stem growth, rather than leaf production (Sawhney *et al.*, 1981).

Calendula officinalis leaves and flowers are edible, and humans have long used it for culinary, medicinal and cosmetic purposes (Sturdivant and Blakley, 1999). Traditionally in countries around the Mediterranean basin and Europe, but more recently in North America, it has been used as an anti-inflammatory agent (Masterová *et al.*, 1991; Akihisa *et al.*, 1996; Bezaková *et al.*, 1996); the triterpenoids which are thought to be responsible for the anti-inflammatory pharmacological effect produced in the flowers (Della Loggia *et al.*, 1994) as well as the flavonoids in the flowers (Masterová *et al.*,

1991). Recent studies have also shown that *C. officinalis* has anti-HIV activity (Kalvatchev *et al.*, 1997), shows heart rate inhibition (Pérez-Gutiérrez *et al.*, 1998), an analgesic in the central nervous system, causing depression of the cardio-respiratory centres (Rahman *et al.*, 1990). It causes reduction of trophic ulcers and their secondary infections (Kartikeyan *et al.*, 1989), shows cytotoxic and anti-tumoral activity (Boucaud-Maitre *et al.*, 1987), and also shows anti-mutagenic activity as a result of saponins in the flowers (Elias *et al.*, 1990). The capitula contain most of the active phytochemicals that are used for medicinal purposes (Sturdivant and Blakley, 1999) and they are present in capitula in their greatest concentration (Bezaková *et al.*, 1996). The most-studied and promising phytochemicals for pharmaceutical use include triterpediol esters (faradiol), triterpene alcohols (helianol, taraxerol, taraxasterol, lupeol, β-amyrin, α-amyrin) saponins, isorhamnetin glycosides and other flavonoids (Della Loggia *et al.*, 1994; Akihisa *et al.*, 1996; Bezaková *et al.*, 1996; Zitterl-Eglseer *et al.*, 1997; Kalvatchev *et al.*, 1997; Pérez-Gutiérrez *et al.*, 1998)

As a medicinal field crop, *C. officinalis* yields 75-110 kilograms of dry capitula per hectare with a market price range of $10-14 Can. per kilogram (Sturdivant and Blakley, 1999). Here I investigated patterns of yield and consistency of product, using *C. officinalis*, grown in a greenhouse using a hydroponic system, with controlled optimization of climate and nutrients, and manipulation of management practices such as simulated foliar herbivory.

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Table 1.1 Medicinal plant Species at Risk (COSEWIC, 2002).

Endangered	Threatened	Special Concern
American Ginseng *Panax quinquefolius*	American Chestnut *Castenea dentata*	American Columbo *Frasera caroliniensis*
Bird's-foot Violet *Viola pedata*	American Water-willow *Justicia americana*	Athabasca Thrift *Armeria meritima* spp *interior*
Drooping Trillium *Trillium flexipes*	Colicroot *Aletris farinose*	Common Hop-tree *Ptelea trifoliata*
Heart-leaved Plantain *Plantago cordata*	Dense Blazing Star *Liatris spicata*	Floccose Tansy *Tanacetum huronense* var. *floccosum*
Hoary Mountain-mint *Pycnanthemum incanum*	Golden Seal *Hydrastis canadensis*	Giant Helleborine *Epipactis gigantea*
Juniper Sedge *Carex juniperorum*	Plymouth Gentian *Sabatia kennedyana*	Green Dragon *Arisaema dracontium*
Prairie Lupine *Lupinus lepidus* var. *lepidus*	Redroot *Lachnanthes caroliana*	Large-headed Woolly Yarrow *Achillea millefolium* var. megacephalum
Small White Lady's-slipper *Cypripedium candidum*	Round-leaved Greenbrier (Great Lakes Plains population) *Smilax rotundifolia*	Macoun's Meadowfoam *Limnanthes macounii*
Southern Maidenhair Fern *Adiantum capillus-veneris*	Small-flowered Sand-verbena *Tripterocalyx micanthus*	Swamp Rose-mallow *Hibiscus moscheutos*
Spotted Wintergreen *Chimaphilia maculata*	Soapweed *Yucca glauca*	Tuberous Indian-plantain *Arnoglossum plantagineum*
Tall bugbane *Cimicifuga elata*	Western Blue-flag *Iris missouriensis*	
Virginian Goat's Rue *Tephrosia virginiana*	Western Spiderwort *Tradescantia occidentalis*	
White Prairie Gentian *Gentiana alba*		

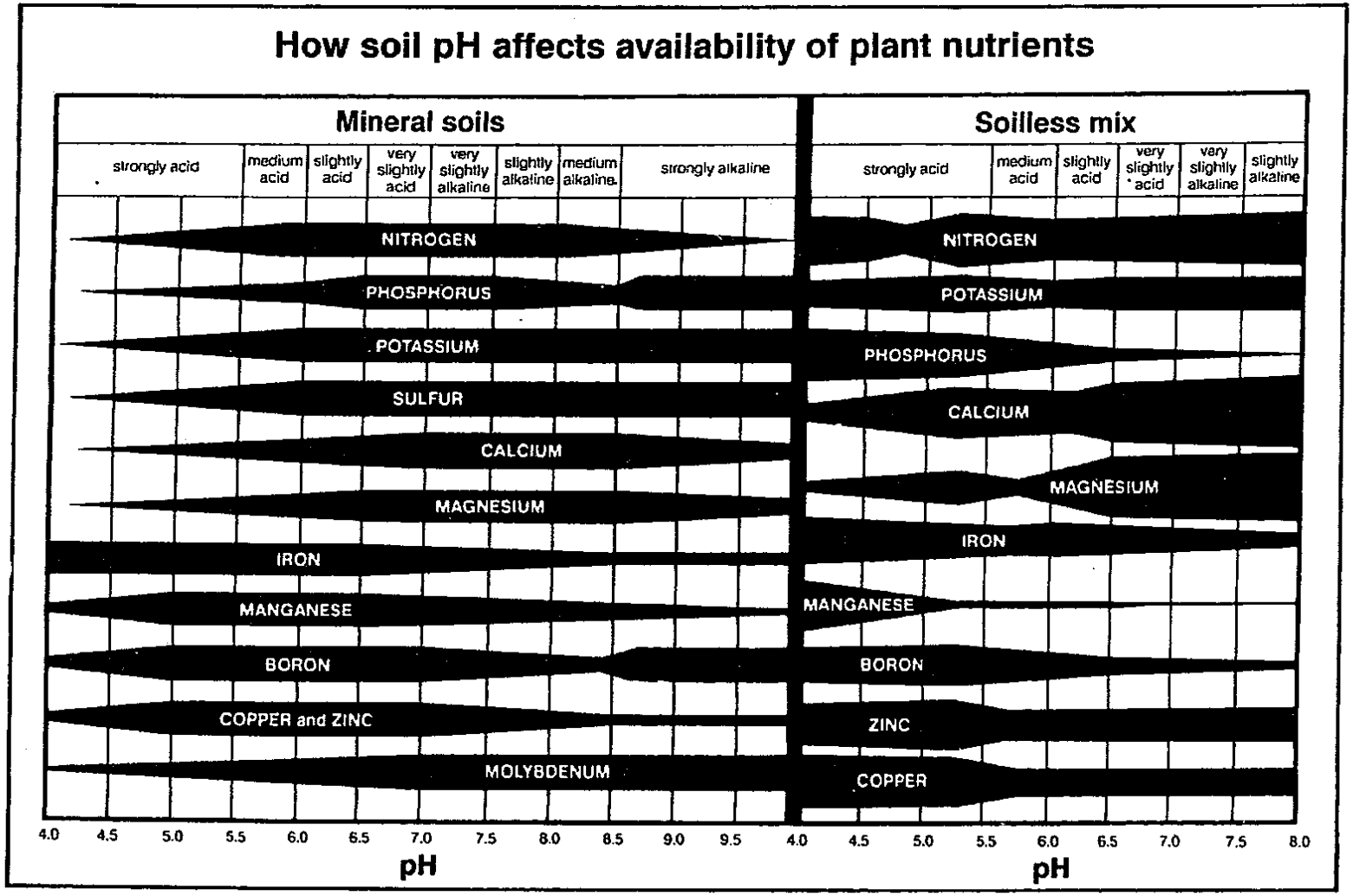

Figure 1.1 Relation between the availability of nutrients and soil on pH (from Plant Products Co. Ltd, 2001).

Figure 1.2. Morphological features of *Calendula officinalis*. A herbaceous annual with a) a hairy stem, alternate leaves and tuberous root with secondary root hairs. The b) capitula comprise of one to several rings of c) ray florets surrounding a central area of d) disc florets. The e) seedhead is comprised of three types of achenes : f) cymbiform (winged); g) vermiform (larval); and h) rostrate (hooked). Only the achenes of the innermost whorl of ray florets, closest to the central disc of the flower, are capable of fruitification. (Adapted from F.E. Köhler, 1887)

Chapter 2

OPTIMIZING PLANT GROWTH AND TISSUE YIELD IN HYDROPONICALLY-RAISED *CALENDULA OFFICINALIS* L.

Introduction

Over the past twenty years in North America there has been a dramatic increase in the level of interest in, and use of, medicinal plants for pharmaceutical applications (Craker, 1999). Between 1999 and 2001 the industry has grown 60% (Papadopoulos and Hao, 2001) and in Canada, imports increased by of $168 million between 1996 and 2000 (Strategis, 2001). To meet this increased demand raw plant material is being purchased from both cultivated and wild sources. This is imperiling populations of some species (Lantz, 2001). Furthermore, there is often little or no regulation/legislation concerning the quality of the plants used for preparing natural health products, or the quality of the source (growing) environment (Rates, 2001). The medicinal benefits to the consumer of 'wildcrafted' or plants collected from the wild may be outweighed by the toxic effects of possible contaminants, including heavy metals, pesticides, radiation, misidentified plants, and undeclared pharmaceutical additives (Januz, 1994; Ahmed, 2001; Abou-Arab *et al.*, 1999; Ernst, 2002). The recent expansion of interest in the use of medicinal plants for personal pharmaceutical use has put pressure on natural populations around the globe, leading to a significant increase in the intensity of unregulated harvesting, which remains the most common way in which medicinal plants are obtained (Ward, 1990). As a result of these concerns, a strong public and regulatory demand for elevated quality, uniformity and safety of medicinal plant products, and the attention to ecological and economic sustainability of wild populations has surfaced. This has all prompted concern for the sustainability and conservation of medicinal plants and their habitats.

One solution to these emergent problems in the market for medicinal products involves controlled environment, greenhouse-based hydroponic cultivation of standardized plant materials to replace wild sources. There are several advantages, including plant purity (via pesticide-, toxin-, and contaminant-free growing conditions), control of secondary metabolite content, and assurance of correct species identification, all as a result of a controlled environment. Economically, such crops would also be an attractive alternative cash crop for growers. As a result, medicinal plants grown in greenhouse hydroponics will be of higher quality and therefore higher market value, particularly if the plant material can be formally certified as organic (Sturdivant and Blakley, 1999).

Given that hydroponic greenhouse cultivation permits the control of growing conditions, the environment can readily be manipulated to optimize medicinal plant growth, and the yield of target plant tissues and specific secondary metabolites. Examples of conditions that can be manipulated include temperature (Mankin and Fynn, 1996; Albright, 1997), light (Mankin and Fynn, 1996; Albright, 1997), carbon dioxide availability (Andriolo *et al.*, 1996), nutrient availability (concentration and/or supply rate) (Huett, 1994; De Rijck and Schrevens, 1998; Papadopoulos, 1998), nutrient solution pH (Siraj-Ali *et al.*, 1987), plant density (Papadopoulos and Ormrod, 1991), grazing (Dyer and Bokhari, 1976) and harvesting methods (Breemhaar and Bouman, 1995). In this study, I applied environmental manipulations to maximize the yield of the target tissue, the flowering head or capitulum, in the medicinal plant *Calendula officinalis,* by varying the phosphorus concentration, nutrient supply rate, and extent of simulated leaf herbivory.

Phosphorus is one of the most important plant macronutrients due to essential its role in the production of nucleotides (Loveless, 1961) as genetic material and as the

energy carrier molecule, adenosine triphosphate (ATP). Phosphorus is particularly important during plant reproduction; several studies have shown that limiting phosphorus supply decreases the production of floral structures (Arnon and Hoaglund, 1943; Shamsi and Whitehead, 1977; Ma *et al.*, 2001) and, conversely, increasing phosphorus concentration stimulates flower production (Besmer and Koide, 1999). Phosphorus is often limiting in natural systems (Raghothama, 2000), especially in Mediterranean climates where *C. officinalis* is native (Duval, 1993).

In order to survive in phosphorus-limited environments, it's suggested that plants adopt a slower growth rate and have a low yield (dry mass) (Gerloff, 1963). Examples include those plants growing in Mediterranean-type soils or other P-limited soils. Where P is the limiting factor for growth and reproduction, an increase in the phosphorus supply in a form available for uptake (orthophosphate, PO_4^-) can increase inflorescence production (Feller, 1995). Feller (1995) increased fertilizer concentrations, including phosphorus independently of the other nutrients, in the P-limited natural growing environment of Dwarf Red Mangrove trees (*Rhizophora mangle*). Higher phosphorus levels resulted in increased inflorescence production, as well as increased production of tannin and phenolic compounds in the leaves. Egle *et al.* (1999) also reported similar results, in four different genotypes of wheat (*Triticum aestivum* L.), whereby increasing phosphorus enhanced grain yields. The increase of P ranged from zero additional P, to 35kg/ha of P, applied to alkaline clay soil in the field.

The control of nutrient supply in hydroponic culture is critical for optimal plant growth. Conventional solution culture techniques, including open, run-through trough systems (Papadopoulos, 1998), often provide conditions in excess: large volumes of solution with nutrient concentrations that are much higher than typical concentrations in

the soil solution are provided, in order to eliminate nutrient/water limitations in the root environment (Papadopoulos, 1998). These conventional hydroponic systems also allow the depletion of nutrients in solution before complete solution replacement and often result in episodic deficiency symptoms and fluctuations in growth rate of the plants (Stadt *et al.*, 1991), both of which compromise plant quality. A steady-state of nutrient supply relative to plant growth, such that the nutrient solution is provided at lower volumes but is more frequently replenished than in depletion-type systems, can be more beneficial (Papodopoulos, 1994; Ingestad and Ågren, 1995) because it supplies nutrients to plants only as needed, and reduce fluctuations in pH, electrical conductivity (reflecting ionic imbalances), and nutrient uptake (Kramer, 1969; Papadopoulos, 1994, 1998). The concentration of P supplied in open, run-through trough hydroponic systems ranges from 29mg/L P/plant for herbs, (Homegrown Hydroponics, 2000) to 62mg/L P/plant for cucumbers (Papadopoulos, 1994).

As a species native to the Mediterranean basin, *C. officinalis* evolved in an environment of limited nutrient and water availability (Ramos, 1998). Mediterranean soils are neutral to alkaline (pH 7.5-8.5) (Carreira *et al.*, 1997), often calcareous in nature with an average EC of 2.8mS/cm (Henkin *et al.*, 1996). They show very low, to deficient phosphorus levels (Cocks and Osman, 1996; Henkin *et al.*, 1996), with different average soil concentrations for dissolved solution P in Mediterranean climates reported between 0.01mg/L- 0.09mg/L at 30cm depth in Spain (Carreira *et al.*, 1997), 2.0mg/L- 8.8mg/L at 10-25cm depth in north-central Portugal (Thomas *et al.*, 1999), and 19.0mg/L at 0-20cm depth in western Australia (Pampolina, *et al.*, 2002). Mediterranean climates occur between 30° and 40° latitude, often on the west coast of continents where cold-offshore currents occur (Yaalon, 1997). Another characteristic of this climate type is wet winters

with dry hot summers; a xerophytic habitat (Yaalon, 1997). As a result of the specific and isolated occurrence of the Mediterranean habitat, a high degree of biotic endemism is found, especially in the flora (Woodward, 1997).

Under experimental conditions, nutrient concentrations and nutrient supply rates are easier to control in hyrdroponic culture since, in soil-based media, some nutrients become attached to soil particles becoming unavailable for plant uptake (Papadopoulos, 1994). In addition, greater amounts of nutrients and water are required in soils due to leaching (Papadopoulos, 1998). For this reason, hydroponic culture using an inert rooting medium, like rockwool, allows a more precisely controlled experiment.

"Overcompensation" is an induced response in plants, involving increased allocation to vegetative or reproductive growth following herbivory or cutting, such that the growth is greater than that of plants that have not been grazed (Agrawal, 2000). Many studies have shown that grazing by herbivores induces the overcompensatory response (McNaughton, 1983; Paige and Whitham, 1987; Nilsson *et al.*, 1996; Papatheodorou *et al.* 1998; Agrawal, 2000). Paige and Whitham (1987) found that *Ipomopsis aggregata* plants browsed by ungulates produced more flowering stalks and a greater mass of leaves, stems, flowers and fruits compared to ungrazed plants. A study by Papatheodorou *et al.* (1998) illustrated that *Quercus coccifera* shrubs grazed by goats had a higher number of leaves per shoot and invested significantly more in below-ground biomass than did ungrazed shrubs. Escarré *et al.* (1996) found supporting evidence that herbivory leads to overcompensation, particularly reproductive overcompensation, for a member of the Asteraceae, *Picris hieracioides*, such that plants that experienced simulated herbivory increased reproductive biomass to a greater extent than did ungrazed plants.

Overcompensation is most likely to occur under optimal environmental conditions (Maschinski and Whitham, 1989; Agrawal, 2000) since plants growing in resource-limited conditions (usually slow-growing species) are not able to acquire adequate nutrients/water to support a sudden increase in growth after herbivory because additional nutrients are not available for uptake (McNaughton, 1983). Gerloff (1963) suggested that plants which grow in nutrient-poor soils, like those growing in P-limited soils, may have adapted to these conditions by having a slow growth rate. This was illustrated by Clarkson's (1967) study of the effects of varying P availability on three *Agrostis* species. When the species were grown in soil that had low P availability and low pH, the only species that established seedlings and maintained a slow, positive, constant growth rate was *A. setacea*, which is found naturally in phosphorus-limited environments. The two other species, *A. stolonifera* and *A. canina* grow naturally in soils in which have more than four times as much phosphorus than the soils that *A. setacea* is found. Clarkson investigated further by growing these three species in solution culture with high P concentration. Again, *A. setacea* exhibited a slow growth rate, slower than that of the other two species. Chapin (1980) cited ten papers in support of the proposal that there is an inherent low rate of growth in plant species that grow in nutrient-limited habitats. Phosphorus is essential in protein synthesis, which is highly correlated with plant growth rates (Loveless, 1961) such that if phosphorus is not available in quantities required to synthesize nucleic acids and ATP to synthesize protein-based structures, then the plant will have a slower growth rate, or further growth will be prevented until phosphorus becomes available. Therefore, species growing in P-limited habitats are not likely to show overcompensation in vegetative or reproductive structures.

Species growing in P-limited habitats are also found to have high concentrations of secondary metabolites (Janzen, 1974; Levin, 1976; Coley, 1983; McNaughton, 1983; Myers, 1988; Woodward, 1997) which have often become used for medicinal or culinary purposes. These secondary metabolites also evolved, it is believed, as a defense response against pests and pathogens invaders, such as herbivores (Siegler, 1996) because they make the plant tissue unpalatable or toxic (Grime, 1977; Chapin, 1980). Slow-growing species found in resource-limited habitats make particularly large investments in anti-herbivore defenses (Coley *et al.*, 1985). This may occur for two possible reasons: the first relies on the premise that slow growing species have long-lived parts (McNaughton, 1983), with large nutrient pools contained within the leaves (Chapin, 1980). The loss of leaves and their nutrient pools by herbivory is of great detriment to slow-growing plants since tissue replacement is slow; therefore, benefit is gained by protecting the plant from herbivory with high tissue concentrations of secondary metabolites (Chapin, 1980).

A second hypothesis that may explain why slow-growing species have high concentrations of secondary metabolites involves surplus carbohydrate and adaptation to a nutrient-limited environment, particularly one limited in terms of nitrogen and phosphorus (Beadle, 1962), which consequently limits the availability of these nutrients within the plant. When both of these elements (N and P) are limited in the environment, a buildup of non-structural carbon occurs, mostly in the leaves, in the form of carbohydrates (Ericsson, 1995). This occurs because although both nitrogen and phosphorus are limited and needed for assimilation in structural growth, carbon is not limiting, and therefore accumulates in the plants. The excess non-structural carbon is used to produce metabolically expensive carbon-based secondary metabolites which act as defense chemicals that in turn deter herbivory (McNaughton, 1983).

In this study, the effects of phosphorus concentrations, nutrient supply rates and simulated herbivory performed as a clippling treatment on the growth and yield of the medicinal plant *C. officinalis*, grown in hydroponic culture, were investigated using a factorial design.

Materials and Methods

Seedling preparation

Plants were raised in the greenhouse at the University of Windsor, from December 2000 to April 2001. Ambient temperature ranged from 22^0C-30^0C and adequate air circulation was ensured by using oscillating fans. Relative humidity ranged from 70-80%. Plants were illuminated for 14 hours a day with 300W high pressure sodium lamps to supplement solar illumination. Seven hundred achenes (the one-seeded fruit of the composites) from several packets from the same seed lot of *C. officinalis* (Richters Seed Company, Goodwood, ON) were individually germinated in 3"x 3" grodan® Stonewool™ (Grodan A/S, Denmark) blocks (also known as rockwool). The blocks were placed in plastic tubs (51cm long x 38cm wide x 11cm deep), 20 blocks to a tub. The 35 tubs were filled with 5L of dechlorinated water, soaking the Stonewool™ blocks, but not immersing the achenes. Tubs were covered with plastic stretch wrap for 48 hours to maintain high humidity during germination, which was mostly complete by the end of the 48 hour period. During the experiment, the water was topped up as needed to maintain the level filled to 2.5cm depth. These conditions were maintained for 25 days before transplanting, which occurred when plants were 25 days old (Day 25).

Transplanting of plants

Thirty-six transparent plastic containers (55cm long by 40cm wide by 15cm deep) were set up as sub-irrigation reservoirs, draped with black polythene around the outside that was tucked underneath, to inhibit algal growth in the nutrient solution. The containers were set up on greenhouse benches in a randomized block design under the same environmental conditions as those used for germination. Bulk reservoirs of nutrient solutions were made up with water that had been dechlorinated for 24 hours, and in these reservoirs any water that evaporated was replaced with dechlorinated water to the marked target volume before the nutrients were added. An air pump (Rolf C. Hagen, Inc., Montreal) was connected to tubing that bubbled air into the reservoirs in order to oxygenate the plants roots. Three hundred and ninety-six seedlings (25 days old) were selected based on their uniform size; each bore two sets of two fully-expanded true leaves. Seedlings, in their Stonewool™ blocks, were placed at random into the containers and a sheet of black polythene, was placed on top with holes cut to fit around the Stonewool™ blocks. This was done to enclose the hydroponic system, and reduce evaporation, algal growth and the risk of microbial infection of the root systems. Plants were tagged, and maintained until harvest, which occurred on Day 126 from planting. On Day 54 a minor outbreak of common whitefly was controlled by applying a soapy water solution every other day to the foliage until the insects were controlled.

Experimental nutrient treatments and clipping treatment

Three different phosphorus concentrations were applied to three groups of 12 tubs each, chosen at random. The twelve tubs, for each P were then randomly split into two

groups that received different nutrient supply rates. Six tubs received one pulse of nutrient solution/24 hours (NSR_{24}) and six received one pulse of nutrient solution/48hours (NSR_{48}). The volume of nutrient solution provided to the plants was increased over the growing period, according to plant growth, but the ratio of nutrient solution in NSR_{24}/NSR_{48} remained the same throughout the experiment (Table 2.1). These volumes were determined in a pilot study carried out in the fall of 1999. Immediately before recharge of the nutrient solutions at the assigned times, any remaining solution was drained off from the tubs. The nutrient supply rates used per plant in this study are less than those provided in conventional hydroponic systems (see Table 2.2). The baseline phosphorus concentrations were 10mg/L (low), the intermediate level of P was 100 mg/L and the high level of P was double this concentration (200mg/L). The phosphorus supplied in all three P concentrations was monopotassium phosphate ($KH_2PO_4^-$, Fisher Scientific Ltd., Fairlawn, NJ), with additional P supplied at the medium and high P level in the form of diammonium hydrogen phosphate ($(NH_4)_2HPO_4$, Fisher Scientific Ltd., Fairlawn, NJ); further P was supplied to the high P level in the form of superphosphate (P_2O_5, Fisher Scientific Ltd., Fairlawn, NJ). All other nutrients were maintained at the same concentration for all treatments. This P level was established based on the results of a pilot study of optimal nutrient concentration carried out in the fall of 1999 (Stewart and Lovett-Doust, 2002, unpublished). The concentrations of macro- and micro-nutrients applied in each phosphorus treatment are listed in Table 2.3. The pH of the solutions was adjusted and maintained between 5.3-5.9 using sulphuric acid, and the EC ranged between 2.17-2.79mS/cm across treatments. These values are within the range that is widely recommended for hydroponic cultivation with rockwool for tomatoes and cucumbers (Papadopoulos, 1991, 1994).

To assess the response of this species to the clipping treatment, each tub that contained one of the three P concentrations and one of the two nutrient supply rates had 10 plants, of which 5 were subjected to clipping and 5 were left ungrazed (control). The clipping treatment consisted of a one-time removal of 50% of leaf area of each leaf, just after the appearance of the first capitulum, on Day 76. All treatment assignments were randomized, as was placement on the benches and location of plants within the tubs.

Parameters measured

Immediately prior to Day 25 of plant growth under the different nutrient treatments, plant height and leaf number was measured for each plant. Interim measurements of leaf number and plant height were made again on Day 57 (following the appearance of the first bud and prior to application of the clipping treatment); on Day 92 (after the clipping treatment); and again on Day 124 (at final harvest) when the plants had experienced the nutrient treatments for 101 days. At that time, leaves, stems and roots were separated, and from each plant fresh mass of each tissue was determined. For each plant the leaves, stems, roots, flowers and buds were gently dried, separately, in an oven at 32^0C for 10 days to constant mass, then the dry mass of each tissue from each plant was determined. Plant height was measured from the root/stem interface to the tallest part of the plant. The capitula were harvested throughout the experiment as each capitulum came to full bloom with its ray florets completely open, (the marketable stage which occurs long before seed set). Growth parameters of the capitula were also measured; their fresh and dry mass, diameter of the disc florets and whole capitulum, and the number of whorls of ray florets were assessed. The day of harvest for each flower

was also recorded to investigate whether certain treatments affected the timing of first flowering or the duration of flower production.

Assessment of water relations

The uptake of nutrient solution from the tubs was indirectly determined over 28 consecutive hours, prior to, (Day 71) and after application of clipping treatment (Day 85), by measuring the height in millimetres of the sub-irrigation solution for all treatment combinations. This height was then correlated with a standard volume curve of known nutrient solution amounts to determine the amount of solution volume used over time. This volume is representative of the collective uptake by all the 11 plants from a tub, since the system was essentially closed, and little evaporation from the surface of the solution could occur. The contribution to nutrient solution made by the blocks themselves was also determined, by measuring the relative water content of the Stonewool™ blocks over 30 consecutive hours, prior to, and after the clipping treatment. This was done by determining the dry mass of a Stonewool™ block without a plant, prior to initiation of the experiment and placing it in the tubs along with the other blocks that contained plants, so that conditions were the same as for blocks growing plants. The blocks were then weighed over time to determine the physical movement of nutrient solution into the blocks and, indirectly, the amount of nutrient solution accounted for by the blocks. Both of these measurements were needed to determine the relationship between nutrient supply rate, solution availability and solution uptake. The amount of nutrient solution present in the blocks and in solution was an indirect indicator of rates of uptake by the experimental plants under each particular set of conditions.

Plant tissue analysis

Twenty samples from each of six treatment combinations underwent tissue analysis for the determination of the amount of the three main macronutrients needed for plant growth; nitrogen, phosphorus, and potassium. The analytical methods used were those indicated for plant tissue in the Model STH Series Combination Soil Outfit Instruction Manual, LaMotte Company (Chestertown, MD), with the corresponding analysis kit (Appendix A).

Statistical Analysis

This was a nested factorial design incorporating phosphorus level, nutrient supply rate, and presence/absence of clipping. Statistical analysis was carried out using SYSTAT version 10.0 (Systat Software Inc., Richmond, CA). General Linear Models were used for comparisons of the plant growth data and plant tissue analysis data, followed, where appropriate, by post-hoc Sheffé's tests. Also, nested ANOVAs were performed to determine within-treatment variance in plant growth data, since each tub contained both grazed and ungrazed plants, which would contribute to within-tub variance. Multivariate analysis of variance with repeated measures was conducted to analyze the water relations data, since these values were measured over a period of time and each value was dependent on the previous values.

Results

Most of the *C. officinalis* achenes germinated within 24 hours of sowing, with germination completed by Day 7 from sowing. Six hundred and twenty-nine seeds germinated out of the 700 achenes planted, giving a germination rate of 90%. Capitula

first appeared on Day 72 from sowing; five in treatment P 100mg/L (NSR_{24}), four in treatment P 100mg/L (NSR_{48}), three in treatment P 200mg/L (NSR_{48}), and two in treatment P 200mg/L (NSR_{24}). At that time no plants were flowering in either of the P 10mg/L treatments. Flowering occurred from Day 72 and was still occurring at harvest (Day 124). A total of 4039 capitula were produced over all treatments. Plants that were provided with the highest concentration of P, at 200mg/L, showed mottled interveinal chlorosis that began on Day 66 on the lower, most mature leaves, progressing up the plant. These chlorotic spots eventually became necrotic. During the application of the clipping treatment, there was an immediate and striking physiological response in the plants whose leaves were cut; their aerial stems, with their terminal buds, arched downward within about 10 seconds. It was noted that plants adjacent to and touching the cut plants also responded in the same fashion, but to a lesser degree. This did not occur in plants that were not in direct contact with the cut plants. The experiment was terminated on Day 124 from sowing.

Plant growth parameters

The results of General Linear Model (GLM) analysis of variance indicated the contrasting phosphorus treatments had a highly significant effects ($p<0.001$ in all instances) on capitula (composite inflorescences), bud and total reproductive dry mass, number of floral buds (to become capitula), number of capitula, total reproductive output, and average dry mass per bud. Phosphorus treatments also significantly affected the number of leaves, plant height, stem dry mass, biomass allocation to roots, capitula, root/shoot ratio and all reproductive structures ($p<0.01$ in each case), and leaf dry mass, and biomass of buds, ($p<0.05$ in each case, Table 2.4).

There was also a highly significant effect of nutrient supply rate on leaf production, plant height, leaf dry mass, dry mass of capitula, and total reproductive dry mass ($p<0.01$, Table 2.4). The numbers of capitula, total reproductive output, root/shoot ratio and the number whorls of ray florets were also significantly affected by nutrient supply rate ($p<0.05$, Table 2.4).

There were significant effects of clipping on the diameter of disc florets ($p<0.01$), and dry mass of capitula ($p<0.05$). Additionally, there was an indication that clipping may affect total reproductive dry mass ($p = 0.052$, Table 2.4), although this did not quite meet the criterion of $p<0.05$ for statistical significance.

Interaction effects between each of the three treatments - P concentration, NSR, and clipping - in all possible combinations were also investigated. There were significant interactions between P concentration and NSR in terms of leaf production ($p<0.001$); leaf, root and bud dry mass, bud production, total reproductive output, number of buds ($p<0.01$); plant height and total biomass, ($p<0.05$, Appendix A).

There were also significant interactions between P concentration and clipping treatment in terms of dry mass allocation to root biomass, ($p<0.01$) and leaf biomass ($p<0.05$, Appendix A).

Interaction effects between clipping and NSR were statistically significant only for allocation to leaf biomass ($p<0.05$, Appendix A). The interaction between all three factors, P concentration, NSR and clipping, significantly affected the diameter of the disc florets of the capitulum and allocation to leaf biomass ($p<0.05$, Table 2.5). Sheffé's post-hoc comparisons of the means were performed to determine which levels of the three treatment variables investigated were significantly different from others. The results of

these comparisons are represented as a letter over each bar of the histograms in Figures 2.1-2.16.

The results of post hoc analyses showed that plants in the lowest P concentration (10mg/L) were the shortest, and had the fewest leaves, with intermediate and high levels of P not differing statistically from each other in either of these respects (Appendix A). The nutrient supply rate that provided the most leaves and the tallest plants was NSR_{48}, where larger quantities of nutrient solution were delivered less often (Appendix A).

Overall, the intermediate P concentration (100mg/L) produced significantly more leaf dry mass than the highest P concentration (200mg/L), although the lowest P concentration did not differ significantly from either the 100mg/L P or 200mg/L P treatment (Fig. 2.1). Overall the nutrient supply rate that provided nutrient solution less frequently, NSR_{48}, produced greater total leaf dry mass than NSR_{24} (Fig. 2.2).

Phosphorus concentration has a significant effect on stem dry mass whereby plants in the intermediate P level has greater mass than plants in the high P level, while plants in the lowest P treatment did not differ significantly from either of the higher P concentrations. (Fig. 2.1)

Percent biomass allocation to roots is clearly responsive to P concentration; at the lowest P concentration, plants had proportionately more biomass in roots than in the two higher P levels, statistically more significant than 100mg/L of P (Fig. 2.3). A greater percentage of biomass is allocated to stems in the treatment with 100mg/L of P than the high P concentration (200mg/L). The treatment with the low concentration of P was not found to differ significantly from the high or intermediate P concentrations. The two highest P concentrations had a significantly greater percent of biomass allocated to reproductive tissues than the lowest P concentration. Post hoc analysis of the ANOVA on

root/shoot ratio demonstrates the significant effects of P concentration and NSR (Figs. 2.4a, b). The highest P level has the highest root:shoot ratio differing significantly from the intermediate P level, but not differing from the lowest P level. The more frequent nutrient supply rate, NSR_{24}, stimulated a significantly higher root/shoot ratio.

The significant effects of P concentration on bud dry mass indicate that 100mg/L of P generated the greatest amount of bud mass, significantly more than both the low and high P concentrations, which do not significantly differ from each other (Fig. 2.1). At 100 and 200mg/L of phosphorus, a significantly greater number of buds and capitula are produced and total reproductive output is greater at these higher P concentrations (Fig. 2.5). NSR affected the number of capitula with NSR_{48} producing significantly more capitula than NSR_{24} (Fig. 2.6).

The average dry masses/ bud, and per capitulum and per reproductive structure were determined by dividing the total dry mass of each category/plant by the total number of each produced/plant. Phosphorus concentration significantly affected the average dry mass/bud such that the intermediate concentration produced buds of significantly greater average mass than the other two P levels (Fig. 2.7).

With respect to capitulum characteristics, the diameter of disc florets was significantly affected by clipping in that ungrazed plants had greater disc diameter than "grazed" plants (Fig. 2.8a). The significant effects of nutrient supply rate on the number of whorls of ray florets show that plants undergoing the NSR_{48} regime developed more whorls of ray florets than those in the NSR_{24} regime (Fig. 2.8b). A post hoc analysis of the effect of the clipping treatment on capitulum dry mass illustrates that ungrazed plants generated more capitulum dry mass than "grazed" plants (Fig. 2.8c).

Differences in the dry mass of capitula/plant and total reproductive dry mass occur at the intermediate and high concentrations of phosphorus, which produced significantly greater total reproductive dry mass than the lowest P concentration (10mg/L) (Fig. 2.9). With respect to nutrient supply rate, NSR_{48} creates significantly more capitulum dry mass (Fig. 2.10) and total reproductive dry mass than NSR_{24} (Fig. 2.10).

The significant interaction between P concentration and NSR was due to plants at the intermediate P concentration with NSR_{48} which produced the greatest number of leaves, number of buds, and total reproductive output, at harvest (Appendix A). Plant height was significantly reduced at the lowest P concentration, with NSR_{24} (Appendix A).

In the post hoc analysis of the interaction between P concentration and NSR, it is clear that at both the low and intermediate P concentrations, NSR_{48} effectively produced significantly greater leaf dry mass than NSR_{24} with the difference being statistically significant at the intermediate P level (Appendix A).

The interaction between P concentration and NSR affects the root dry mass such that at 100mg/L of P (the middle concentration) the less frequent nutrient supply rate with more solution volume produce greater root mass than a smaller volume of solution that is provided more often (NSR_{24}) (Appendix A). On the other hand, at the high P concentration, the outcome was reversed with NSR_{24} producing greater root dry mass than NSR_{48}.

A significant interaction between P concentration and NSR occurred when P was at its intermediate concentration where NSR_{48} generated a significantly greater number of buds, bud mass and greater total reproductive output than at NSR_{24} (Appendix A).

Phosphorus concentration and NSR interact to significantly affect the total biomass at the intermediate P concentration where NSR_{48} produces greater plant biomass

than NSR_{24} (Fig. 2.11). Allocation to leaf biomass is significantly affected by several interacting factors. The interaction between NSR and clipping (Fig, 2.12a) and the interaction between P and clipping shows that "grazed" plants allocate a greater amount of biomass to leaves at NSR_{24} and the lowest phosphorus concentration, respectively (Figs. 2.12c).

The interaction between P concentration and clipping is such that, only at the lowest P concentration and in the absence of "grazing", plants allocate more biomass to roots than did "grazed" plants (Fig. 2.12b). When all three variables are examined, the combination of low P levels, NSR_{24}, and "grazing" causes plants to allocate a greater amount of biomass to leaf tissue.

Water Relations

The amount of nutrient solution in the treatment containers was measured over a 28-hour period, before (Day 71) and after (Day 85) the clipping treatment was applied. This quantity is representative of the amount of nutrient solution used by the plants in each treatment, and was used as an indirect measure of plant uptake of water and nutrients. For plants measured before clipping occurred, repeated measures ANOVAs showed statistically significant differences in nutrient solution volume leftover for the effect of NSR, time, and the interaction of NSR with time ($p<0.001$, Fig. 2.13a, Table 2.6, Appendix A). Following the clipping treatment, statistically significant effects were seen only for time and the interaction between time and NSR ($p<0.001$, Fig. 2.13b, Appendix A).

The relative mass of unoccupied Stonewool™ blocks was used as a measure of nutrient solution availability at a given point in time to the plant since the weight of the

blocks measured how much solution was being retained and available in the capillary spaces of the block. This was measured before and after clipping over a 30-hour period. Repeated measure ANOVAs indicated a statistically significant effect of time ($p<0.001$), NSR and the interaction between time and NSR ($p<0.01$) on the relative mass of the blocks prior to clipping (Fig. 2.14a, Table 2.6, Appendix A). Following clipping, statistically significant effects on the relative mass of the blocks were seen in NSR, time, the interaction of time and P concentration, the interaction of time and NSR, ($p<0.001$); the combination of time, NSR and P concentration and time ($p<0.01$ Fig. 2.14b, Table 2.6, Appendix A). The significant effect of the time factor indicates a change in the mass of the blocks over the time period they were measured.

Tissue Analysis

Analyses of variance were conducted on the concentrations of the three main macronutrients, phosphorus, nitrogen and potassium in leaf tissue. The results showed a marginally significant difference for the concentration of nitrogen with respect to the nutrient supply rate ($p = 0.053$) and a significant interaction between NSR and P concentration ($p < 0.01$, Fig. 2.16a, b, Table 2.7). The nutrient supply rate that provided lower volumes of nutrient solution more frequently led to a lower leaf tissue nitrogen concentration than the less frequent NSR. The significant interaction of NSR and P concentration for N concentration in leaf tissue occurred at the highest P level with NSR_{48} maintaining a higher N concentration in leaf tissue than NSR_{24} (Table 2.7). Although significant differences were not found in the leaf tissue concentration of P, the trend for the effects of P concentration in solution indicates that the plants provided with the lowest P concentration in solution had the lowest mean P concentration in the leaf tissue, while

the other two P levels had relatively similar amounts of P concentration in leaf tissue. There were also no statistically significant differences in leaf tissue with respect to K concentration; the observed trend for the effects of P concentration in solution illustrated that plants provided with the highest P level had the lowest mean value for leaf tissue K concentration. The plants provided the highest P concentration in solution had the lowest mean value of leaf tissue nitrate nitrogen concentration (Appendix A).

Discussion

The germination rate in this study was 90%. This is in contrast with soil-sown seeds of *Calendula officinalis* germinated in a greenhouse, which are reported to have a low rate of germination (Duval, 1993) and with other germination rates for soil-sown greenhouse seeds found to be between 63-68% (Bass, 1980). Capitula first appeared on Day 72 of plant growth which is a slower time to bloom than the fastest flowering, reported soil sown achenes, of 62 days (Zimmer, 1989) and our previous finding for hydroponically sown achenes which began to flower at 53 days (Stewart and Lovett-Doust, 2002, Canadian Journal of Plant Science). While the clipping was occurring, the plants responded physiologically by the arching downward of stems that had terminal buds. The plants adjacent to and touching the cut plants, that had not been cut themselves, responded in the same physiological manner, but to a lesser degree. This suggests that the damaged (cut) plants may be induced to instantly release a volatile chemical in response to herbivory that can act as a signal to plants that are touching them in a manner analogous to allelopathy (Basile *et al.*, 2000), with allelopathic substances being described as either volatile terpenes or phenolic compounds (Harborne, 1988).

Phosphorus concentration, nutrient supply rate, clipping and the interactions of these factors all significantly affected various parameters of growth and development in *C. officinalis*. Water availability, represented by solution volume and solution content within the Stonewool™ blocks, and the concentrations of nutrients found in leaf tissue were also significantly affected. The absolute yield of the target tissues (capitulum) increased with increasing phosphorus concentrations, with the optimal concentration for total number of capitula being at 200mg/L of P (Fig. 2.7). Phosphorus has been shown to be a critical nutrient for plant growth, associated, in particular, with inflorescence production (Egle *et al.*, 1999). In *C. officinalis*, even though the optimal mean yield was obtained at 200mg/L, there were no significant differences in the percent allocation of biomass of reproductive output between the intermediate and high P concentrations (Fig. 2.5). At the intermediate P level, 100mg/L is supplied to 11 plants, averaging a supply of 9.1mg/L/plant. This concentration is significantly lower than that used in typical hydroponic cultivation (from 29mg/L P/plant for herbs (Homegrown Hydroponics, 2000) to 62mg/L P/plant for cucumbers (Papadopoulos, 1994). Borch *et al.* (1998) found that plant quality in Impatiens (*Impatiens wallerana* Hook. f. 'Impulse Orange') and Marigolds (*Tagetes patula* L. 'Janie Tangerine') improved, and plants were more drought resistant, at lower P concentration than in conventional hydroponic solution. The law of diminishing returns would predict that increasing fertilizer concentrations will increase plant growth and reproduction up to a threshold, at which point growth then reaches a plateau with no further increase in growth with increasing fertilizer (Chrispeels and Sadava, 1994).

The evolution of *C. officinalis* in dry, infertile soils may result in a low absorption rate of P per plant and/or a low requirement for P (Clarkson, 1967; Chapin, 1983);

therefore *C. officinalis* may show a weak growth improvement response to increasing external P concentrations. Clarkson (1967) illustrated the low requirement for P in *Agrostis setacea*, which is found in naturally P-limited soils. When he grew this species in soil with low P availability, with two other *Agrostis* species that are adapted to natural environments with higher soil P availability, he found only *A. setacea* established seedlings and maintained a slow (but positive) rate of growth. He also showed the minimal response to increasing external concentrations of P for *A. setacea* when he grew the three species in solution culture with high concentrations of P. Even though the P availability was greater than that found in the natural soils for *A. setacea*, it still maintained a slow rate of growth in comparison to the other two *Agrostis* species. A similar pattern may explain the lack of statistically significant differences between high and intermediate P concentrations in this experiment.

The yield of capitula was also optimized with the nutrient supply rate that provided a larger volume of nutrient solution, less frequently (Fig. 2.8). This finding supports the perspective that a large steady supply of nutrients can actually be detrimental to plant growth and reproduction. Phosphorus can be taken up by luxury consumption, even though a plant may have enough for adequate current growth (Lajtha and Klein, 1988), however luxury consumption can lead to toxicity effects such as inhibited growth and chlorosis. Luxury consumption is the uptake of nutrients in excess of immediate growth requirements (Chapin, 1980). High P concentrations have been found to lead to P toxicity at 240 mg/L in tomatoes (H. A. Mills, University of Georgia, Athens, Georgia, pers. comm.). Typically, the slow-growing species of infertile soils absorb nutrients in excess of growth requirements during nutrient flushes, which is referred to as luxury consumption (Chapin, 1980), which in this experiment is perhaps best represented by

NSR_{24}. Both Clarkson (1967) and Christie and Moorby (1975) showed luxury consumption of phosphorus by plants that are naturally adapted to P-limited environments, compared to those that are adapted to environments with higher P availability, when they were all grown with greater P availability than in the P-limited environments. These slow-growing species showed greater shoot and root phosphorus concentration, respectively, than the faster growing species adapted to nutrient-rich environments.

Chapin (1980) suggested that the nutrients are absorbed in excess of immediate growth requirements during these nutrient flushes to support growth when the soil P is exhausted. This increases the chances for toxicity when the slow-growing species are transplanted to, or grown in nutrient-rich environments, as we may have seen in this study when plants at the highest P concentration (200mg/L) showed interveinal chlorosis (which began on the lower, most mature leaves). These symptoms then progressed up the plant with the chlorotic spots eventually becoming necrotic. This symptom is usually interpreted as zinc deficiency. High tissue phosphorus concentrations have been shown to induce zinc deficiency due to the formation of soluble zinc phosphates (Papadopoulos, 1994), which can be indirectly caused by phosphorus toxicity. This means that for cultivation of *C. officinalis*, conventional P levels used in open run-through trough hydroponic systems and even soil-based media can be decreased, lowering fertilizer costs and reducing release of surplus P into the environment (Papadopoulos, 1998) when growing plant species that are naturally adapted to P-limited environments. Plants adapted to low-nutrient soils tend to have low nutrient requirements (Clarkson, 1967), such that lower levels of P are optimal for normal growth. Mediterranean soils are typically low in nutrient concentrations, especially phosphorus (FitzPatrick, 1980) and are

dominated by sclerophyllous plants, like *C. officinalis* (Woodward, 1997), that have low nutrient requirements (Loveless, 1961).

The effect of P concentration on the percent allocation of biomass to reproductive structures is such that plants in the two highest P levels allocated proportionately more biomass to reproduction than did plants grown at the lowest P level (Fig. 2.5). Phosphorus levels of 10mg/L are not sufficient to support high levels of reproductive output or large reproductive structures. Overall, increasing phosphorus concentrations to the intermediate level (100mg/L) increased both above-groud vegetative and reproductive growth, which was the aim of the study, at the expense of root growth, which increased for 10mg/L of P and 200mg/L of P. Figure 2.3 illustrates the tradeoff between percent root biomass allocation and the percent biomass allocated to other tissues, where greater root mass is associated with to a decrease in percent biomass allocated to reproductive tissues (at 10mg/L) or a decrease in percent biomass allocated to leaf tissue (at 200mg/L), which is undesirable with regard to the aim of the study.

Optimal above-ground, and reproductive absolute dry mass was achieved at the mid-level phosphorus concentration, of 100mg/L (Fig. 2.3), even though a greater number of capitula were produced at 200mg/L. This effect is amplified by the interaction of nutrient concentration with nutrient supply rate with greatest vegetative and reproductive dry mass being achieved at 100mg/L and NSR_{48}, (with significantly less biomass being allocated to roots). In the desert shrub, *Larrea tridentata*, Lajtha and Klein (1988) similarly found that increasing P concentration caused a 2.5-fold increase in biomass. Root growth is often enhanced under low nutrient conditions (Chapin, 1980) as it is interpreted as a response to the need to "forage" for nutrients in the soil. In this study, root dry mass was greatest at the highest and lowest P concentrations; this effect is further

illustrated by the high root/shoot ratios at the highest and lowest P concentrations. This suggests that plants provided with the lowest P concentration increased their root mass in order to access more P, to support plant growth. In the highest P concentration, although plants were supplied with ample P for plant growth, uptake was often complicated by the fact that ions compete with each other for active transport sites. For example, potassium and phosphorus compete with each other for uptake by plants (Papadopoulos, 1994; Feller, 1995), as well as competing with nitrogen ions (Papadopoulos, 1994). Feller (1995) showed that a prolonged enrichment of P, in a typically P-limited environment, lead to an increased rate of photosynthesis and greater net assimilation rates in Dwarf Red Mangrove (*Rhizophora mangle*). This in turn increased demands for K, effectively diluting available K in the plants and decreasing its concentration in plant tissues. In the present study, there was a similar decrease in the amount of nitrate nitrogen and K in leaf tissue at the highest P concentration, at 200mg/L. In contrast, De Magalhaes *et al.* (1998) found, in corn (*Zea mays*), during P starvation, nitrate uptake decreased; when P levels were restored, nitrate uptake resumed. The results showed that maximum P and NO_3^- concentrations in leaf tissue were achieved at 100mg/L of P. This may also contribute to the optimal vegetative and reproductive dry mass production noted at this intermediate P concentration, since nutrient-stressed plants reduce absolute allocation to reproduction (Feller, 1995).

When comparing the percent allocation of biomass with absolute biomass of each tissue (Fig. 2.3 and 2.5) we see that, although there is no significant difference between P concentrations in terms of root biomass, there was a significant difference between P concentrations in terms of the proportion of biomass that is allocated to the roots, with the lowest P level having the greatest proportion of root biomass. This suggests that across P

treatments, the absolute biomass of the roots remains relatively constant, when looking at the plant as a whole in terms of allocation, a greater proportion of plant biomass is allocated to the roots of the plants that have the lower P supply. A proportionate increase in biomass allocated to roots under nutrient stress is supported by several studies, reviewed by Chapin (1980). The opposite pattern is seen in leaf tissue, where there is a significant difference between P concentrations in terms of absolute leaf biomass with 100mg/L P producing the greatest amount and no significance difference between P concentrations for the proportion of biomass allocated to leaves. Providing adequate amounts of P appears to increase the absolute biomass of leaf tissue, but altering concentrations of nutrients does not appear to affect the proportion of biomass allocated to leaves, possibly because nutrient reduction has less of an effect on percent allocation (secondary control) than do the primary effects of plant hormones that control plant growth (Chapin, 1980). The two highest P concentrations allocated the greatest proportion of biomass to reproductive tissues, which are the target tissues and of greatest interest, and the median P concentration also allocated the greatest percent biomass to plant stems, possibly to support the increased reproductive mass. Overall, 100mg/L of P provides the optimal situation with the greatest proportion of biomass allocated to reproductive structures and the least amount of percent biomass "wasted" on root structures.

The nutrient supply rate that provides a larger volume of nutrient solution less often, NSR_{48}, also generated the greatest amount of each plant tissue being measured. This further illustrates the detrimental effect of luxury uptake of excess nutrients on plant growth. There is a significant relationship between resource availability and the amount of biomass allocated to vegetative growth and reproduction (Bazzaz, 1987). NSR_{24}, over

all P concentrations, also represented a certain degree of water stress because water/nutrient availability fluctuated and there were times when plants had absorbed all the solution and the tubs no longer contained nutrient solution (Fig. 2.13a, b). A result of this stress would be seen in terms of a reduction of dry mass in vegetative and reproductive tissues, which was the case in the present study. The degree of water stress is inversely related to the rate of nutrient supply in solution culture. Conversely, Busso *et al.* (1998) and Biagorri *et al.* (1999) suggest that plants which are more resilient to drought-like conditions tend to allocate equal or more dry mass to reproductive organs during times of water stress than at times when nutrients/water are available. Busso *et al.* (1998) showed that in *Medicago minima* and *Erodium cicutarum*, plants under water stress allocated greater total dry mass to reproduction. Both of these plants, like *C. officinalis*, are naturalized annual species typically found in a semi-arid habitat. When considering the native environment of *C. officinalis*, which has little and infrequent water supply during the growing season, it would then follow that NSR_{48} better represents its natural environment, as oppose to NSR_{24}. If this is the case, then NSR_{24} would represent water stress not NSR_{48}, which would then support Busso *et al.* (1998) and Biagorri *et al.*'s (1999) findings with respect to dry mass allocation to reproductive tissues.

The nutrient supply rate that maximizes root/shoot ratio is the NSR that provides less nutrient solution volume more frequently (NSR_{24}). In this case it is possible that greater root production occurs because if luxury consumption of P occurs with this NSR, as it does with slow-growing species adapted to nutrient-limited environments that experience nutrient flushes (Chapin, 1980), then the high levels of P would cause zinc deficiency (Papadopoulos, 1994), as well as compete for uptake with potassium (Papadopoulos, 1994; Feller, 1995) and nitrogen ions (Papadopoulos, 1994). This would

result in a greater effort to absorb these nutrients, which can be achieved by an increase in root growth (Chapin, 1980). In these conditions of decreased nutrient availability due to ionic competition, biomass allocation to roots is increased, when the biomass could have otherwise been used to produce other photosynthetic or reproductive tissues, with leaves having the capacity for greater energy production (Konings, 1989). This also suggests why dry mass of leaves, plant height, and leaf number, as well as reproductive measures (dry mass and output) may be greater for NSR_{48}, which seems more likely than the possible effect of water stress from this nutrient supply rate.

An interesting effect of nutrient supply rate was that more whorls of the outer ray florets were produced with the optimal nutrient supply rate for reproductive growth, NSR_{48}, which provided nutrients less frequently (yet maintained a more constant water/nutrient supply) (Fig. 2.11). This suggests that plants that are provided optimal amounts of nutrients for vegetative and reproductive growth can also afford to increase the size of the floral display and achene production. In *C. officinalis,* the disc florets that make up the central portion of the capitula are perfect (hermaphrodite), but only have male function, while the ray florets are pistillate (female) and produce achenes (Gardocki *et al.*, 2000), but only in the innermost whorl of ray florets (Jost, 1983). An increase in floral display is likely to be associated with male function, in that large floral displays are associated with selection to export pollen by attracting pollinators (Corbet *et al.*, 2000). The selection to export pollen also supports the tendency towards cross-pollination in this protandrous (stamens ripen first) species (Harder, 1965). Also, this increase in the number of whorls of ray florets may provide a greater opportunity for these plants to pass on their genes through successful female function with greater seed production supported by the greater vegetative biomass. For greater achene production to occur, more

resources will be required to support fruit production (Freeman and Harper, 1980). This suggests that even though NSR_{48} provides nutrients less frequently, it allows superior, possibly more efficient, utilization of the nutrients available to the plants over that time period. Nutrients supplied less often will not necessarily be a negative pressure in this study, especially since the solution in NSR_{48} remains available longer and allows a more constant nutrient uptake. Novak *et al.* (1999) also observed a strong environmental influence on number of whorls of ray florets of eight cultivars of *Calendula officinalis,* grown in two different locations in Austria.

Gardocki *et al.* (2000) reported changes in the frequency of male and female flowers of *Calendula micrantha,* grown in soil with different watering frequencies. The occurrence of female flowers dramatically increased with increasing watering frequency with the wet pots that received water every day having the greatest number of female flowers. These examples illustrate that the frequency of nutrient and water pulses is as important, if not more important than nutrient concentrations, to optimal plant growth (Lapointe, 1985) particularly with respect to native habitat type for *C. officinalis*, since it is nutrient limited. This makes slow-growing *C. officinalis* more sensitive to nutrient pulses.

Clipping was found to decrease the total dry mass of capitula and the diameter of the functionally male disc florets (Fig. 2.8). These two observations combined suggest that as clipping adjusts the allocation of disc florets, by decreasing the diameter of the whole disc (either by producing fewer disc florets or smaller ones) the mass of the whole capitulum decreases. In this case foliar clipping is, specifically, causing a decrease in the production of hermaphrodite (but functionally male) flowers in the capitulum, and therefore a decrease in male function/pollen export. Similarly, Rodriguez and Brown

(1998) found a decrease in the biomass of reproductive organs and reproductive allocation under herbivory. Escarré *et al.* (1995) found that in two other members of the Asteraceae (*Crespis foetida* and *Crespis pulchra*), late defoliation, which occurred at approximately 60 days after germination, decreased sexual biomass (mass of capitula) significantly. Lehtila and Strauss (1999) found that male reproductive characteristics are more affected than female characteristics by herbivory in radish (*Raphanus raphanistrum*), such that petal size (which is representative of male function in this species), and thereby the floral display, decreased with herbivory. Similarly, Freeman and Harper (1980) found a differentiation in the prevalence of the sexes in the dioecious species *Atriplex confertifolia* (shadscale) grown in grazed and ungrazed pastures. Male plants were found to be over-represented in the patches that excluded grazing while females had higher prevalence in grazed patches.

Foliar clipping, simulated by 50% leaf lamina removal, considered as an effect alone, did not induce overcompensation in vegetative growth or reproductive output. However, plants naturally adapted to nutrient stress tend to allocate more carbon to carbon-based defenses, such as phenolics, instead of showing growth enhancement under herbivory, due to the fact that nutrients are limiting and the plants already have a slow growth rate (McNaughton, 1983).

There was an apparent tradeoff between root and leaf biomass distribution. Although overcompensation does not occur when herbivory acts independently, at low concentrations of P, combined with the effect of the clipping treatment, caused "grazed" plants to increase biomass distribution to leaves and decreased biomass allocation to roots (Fig. 2.12b). At this P level, the tradeoff occurs and there was overcompensation in terms of leaf production. McNaughton (1983) similarily found a reallocation of biomass from

storage organs (in his study, these were roots) to other vegetative tissues. This is most likely due to the need to replace photosynthetic tissues that were removed during the clipping treatment and to ensure sufficient energy capture for these plants to reproduce successfully. "Grazed" plants also experienced an increase in leaf biomass when the clipping treatment was combined with NSR_{24} (the rate that provides nutrient more frequently in smaller quantities) (Fig. 2.12c). As discussed previously, at NSR_{24}, luxury uptake of P by the plants likely occurs in response to the increased frequency of nutrient availability, which causes competition for uptake with other ions. Such conditions may stress the plants, in addition to the stress caused by herbivory. It appears that more than one applied stress is needed to stimulate the response of statistically significant proportionate reallocation in plant tissues. Consequently, when all three stresses are applied: low P concentration and, frequent NSR, combined with clipping, the result is a greater increase in leaf biomass for "grazed" plants than in the treatments that experience only two stresses of these at the lowest P concentration and NSR_{24}.

Table 2.8 summarizes, for each P concentration, the comparison of the means of growth measurements of field and hydroponic plants grown under floating raft culture. Values from *C. officinalis* from various studies are compared with some of the results from this study. Floating raft hydroponic culture, used by Dorais *et al.* (2001), is a closed recirculating system that provides large volumes of nutrient solution to plants that is topped up as needed and complete solution replacement occurs approximately once a month. The plants are placed in cut-out holes in styrafoam floats that float on top of the nutrient solution with the roots submersed in the nutrient solution. Our optimal P concentration (100mg/L) showed a 10-fold or greater production of leaves, taller plants and greater dry mass in terms of all growth measurements compared to field-grown and

floating rafts plants. This is most likely due to greater control of the environment and resources in shallow culture hydroponic cultivation used in this study. When comparing the means for the optimal P concentration for shallow culture hydroponics to the floating raft (deep culture) technique, the results of this study illustrate a greater number of leaves produced, taller plants, and greater dry mass of roots; contributing to the greater root shoot ratio for shallow culture hydroponics. The floating raft technique, which provided approximately half the amount of P to plants provided in shallow culture technique, produced greater dry mass of aerial parts and flowers, but the mean values showed a large standard error. It is possible that although this study indicated 100mg/L of P is optimal, amounts of P between 10-100 mg/L that were not investigated in this study, may further enhance the production of target tissues. The difference may also be a result of the type of hydroponic system demonstrated, whereby the floating raft technique may require less P supply for optimal growth than the shallow culture technique, but further studies would need to be done to show this.

Measurements of water relations indicate that NSR affected the solution volume significantly before clipping, but not following the clipping treatment. Prior to defoliation, the solution in NSR_{48} is used up gradually over the time period before nutrient recharge. This provided the plants with a more consistent and steady uptake of water and nutrients, than in NSR_{24} which is recharged with new nutrient solution more frequently. An infrequent nutrient supply was associated with less fluctuation in nutrient concentrations and EC, reducing osmotic stress on the plants. This may reflect the fact that *Calendula officinalis* is adapted to soils that do not experience frequent renewal of nutrients and water. Hydroponically-grown plants that remain under a steady nutritional state over time show minimal deficiency symptoms, even though nutrients may be limited

(Ingestad and Lund, 1986). This translates into more consistent growth rates and optimal conditions for maximal plant growth since the solution uptake is representative of nutrient solution use, and therefore of plant growth. Following clipping, there were no significant differences between nutrient supply rates in terms of the amount of solution absorbed by the plants. As expected, time had a significant effect on solution volume, prior to and following clipping, since the change in volume represents nutrient uptake, as well as the interaction of time and NSR. Also, there was more variance because both "grazed" and ungrazed plants were represented within each.

The relative mass of the Stonewool™ blocks indicated that nutrient solution remained available even after the solution volume in the containers had declined to zero, since some solution always remained held in the capillary spaces of the blocks. Solution availability can be associated with plant growth in the same manner as solution volume, such that the greater the mass of the blocks, the greater the solution availability maintained within the blocks, and therefore available for plant uptake, supporting plant growth. The results indicate differences between NSRs prior to and following the clipping treatment. Interestingly, a greater amount of solution was retained in the blocks exposed to NSR_{48} than in the blocks experiencing NSR_{24}. In the seaweed *Gracilaria tikvahiae*, Lapointe (1985) found that the uptake rate of P increased with decreasing frequency of nutrient solution supply. If our results are parallel to these findings, then in NSR_{48} we can assume P uptake would be greater. Predictably, time and the interaction between time and NSR affect the actual quantity of solution held in the block, both before and after clipping, showing a decrease in relative mass of the solution in the blocks over time as solution uptake continues over time.

Conclusions

Higher phosphorus concentrations increased reproductive output (number of capitula plus buds), absolute reproductive dry mass, and proportionate distribution of biomass to reproductive tissues, with optimal results (greatest yield of capitulum tissue) being achieved at 100mg/L. Doubling the phosphorus concentration to 200mg/L did not provide significantly more of the target tissue; it would therefore be a wasted input for growers to continue to increase phosphorus fertilization beyond the 100 mg/L level. The most appropriate nutrient supply rate, which provided the greatest amount of target tissue from *C. officinalis*, was the supply rate that provided a larger volume of solution, less frequently with more consistency of solution availability (NSR_{48}). This also has applications for the grower, since less frequent labour inputs would be required. Fifty-percent, one-time, partial defoliation did not induce overcompensation in reproductive tissues, but did provided greater stress to the plants, reducing inflorescence size. An overcompensation response in terms of increased leaf tissue only occurred when clipping was combined with the lowest P concentration.

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Table 2.1 Volume of nutrient solutions provided at each nutrient supply rate interval.

Day from sowing	**Volume NSR_{24}**	**Volume NSR_{48}**
Beginning of varying NSRs (Day 40 - 63)	500ml	1000ml
Day 64 – 88	1000ml	2000ml
Day 89 – 105	1300ml	2600ml
Day 106 – 124	1000ml	2000ml

Table 2.2 Nutrient supply rates used in this study and that was used in other hydroponic cultivation systems.

Calendula officinalis		Supply rate (L/plant/day)	Cucumber- fall crop[1]	Supply rate (L/plant/day)	Tomato- fall crop[2]	Supply rate (L/plant/day)
Day of growth	Developmental stage		Day of growth		Day of growth	
1-8	germination	0.025	1-7	0.8	1-28	0.6
9-23	two-leaf stage	0.050	8-14	1.2	29-56	0.8
24-39	four-leaf stage	0.050	15-21	1.0-2.0	57-84	1.0
40-63	leaf rosette	0.045	22-56	2.0-3.0	85-98	0.8
64-88	first capitula	0.09	57-end	1.0-2.0	99-end	0.4
89-105	full reproduction	0.12				
106-124		0.09				

[1] taken from Papadopoulos, 1994
[2] taken from Papadopoulos, 1998

Table 2.3. Nutrient concentrations of feeding solutions in each phosphorus treatment.

Nutrient	Low P treatment (mg/L)	Intermediate P treatment (mg/L)	High P treatment (mg/L)
P	10.0	100.0	200.0
N	200.0	200.0	200.0
K	200.0	200.0	200.0
S	138.0	138.0	138.0
Ca	100.0	100.0	100.0
FeEDTA	70.0	70.0	70.0
Mg	50.0	50.0	50.0
Mn	20.0	20.0	20.0
B	13.0	13.0	13.0
Mo	0.6	0.6	0.6
Zn	0.5	0.5	0.5
Cu	0.1	0.1	0.1

Table 2.4. General Linear Model ANOVA results of main effects of P, NSR, and clipping on growth.

Growth Parameter	Source of variation: P				Source of variation: NSR				Source of variation: Clipping			
	df	MS	F ratio	P value	df	MS	F ratio	P value	df	MS	F ratio	P value
Number of leaves	2	70469.7	7.73	p <0.01	1	72179.1	7.91	p <0.01	1	419.9	0.05	NS
Plant height	2	792.2	7.05	p <0.01	1	927.7	8.26	p <0.01	1	0.7	0.006	NS
Dry mass roots	2	1.1	1.30	NS	1	0.2	0.24	NS	1	0.03	0.04	NS
Dry mass stem	2	13.4	5.93	p <0.01	1	5.6	2.49	NS	1	6.4	2.85	NS
Dry mass leaves	2	11.2	4.01	p <0.05	1	28.9	10.35	p <0.01	1	1.2	0.43	NS
Dry mass buds	2	0.1	12.38	p <0.001	1	0.04	3.81	p =0.052	1	0.000	0.01	NS
Dry mass capitula	2	2.5	8.90	p <0.001	1	2.6	9.18	p <0.01	1	1.1	4.00	p <0.05
Dry mass total reproduction	2	3.5	10.79	p <0.001	1	2.7	8.40	p <0.01	1	1.2	3.81	p =0.052
Total biomass	2	13.2	0.96	NS	1	35.2	2.56	NS	1	5.1	0.37	NS
% Root mass allocation	2	0.03	6.11	p <0.01	1	0.000	0.03	NS	1	0.000	0.09	NS
% Stem mass allocation	2	0.01	2.57	NS	1	0.004	1.43	NS	1	0.005	1.72	NS
% Leaf mass allocation	2	0.002	0.29	NS	1	0.002	0.28	NS	1	0.017	2.93	NS
% Bud mass allocation	2	0.000	3.43	p <0.05	1	0.000	0.91	NS	1	0.000	0.19	NS
% Capitula mass allocation	2	0.007	5.54	p <0.01	1	0.000	0.01	NS	1	0.002	1.26	NS
% Reproductive mass allocation	2	0.1	6.79	p <0.01	1	0.000	0.10	NS	1	0.001	0.94	NS
Root/shoot ratio	2	0.08	5.18	p <0.01	1	0.09	5.40	p <0.05	1	0.003	0.17	NS
Number of buds	2	346.8	8.94	p <0.001	1	71.9	1.85	NS	1	25.8	0.67	NS
Number of capitula	2	729.5	13.30	p <0.001	1	223.3	4.07	p <0.05	1	141.4	2.58	NS
Total reproduction	2	1958.2	16.52	p <0.001	1	535.9	4.52	p <0.05	1	256.6	2.17	NS
Average mass/ bud	2	0.002	12.52	p <0.001	1	0.000	0.99	NS	1	0.000	0.004	NS
Av'g mass/capitulum	2	0.014	1.97	NS	1	0.017	2.45	NS	1	0.023	3.32	NS
Average mass/ reproductive structure	2	0.003	2.48	NS	1	0.003	2.54	NS	1	0.001	1.25	NS

Table 2.5. General Linear Model ANOVA results of main effects of treatments, P, NSR and clipping, P*NSR*clipping, Rep (P*NSR), clipping*Rep (P*NSR) on characteristics of capitula.

Capitula characteristics	Source of variation: P				Source of variation: NSR				Source of variation: Clipping			
	df	MS	F ratio	P value	df	MS	F ratio	P value	df	MS	F ratio	P value
Diameter of whole capitulum	2	0.4	0.83	NS	1	0.01	0.03	NS	1	1.0	2.30	NS
Diameter of disc florets	2	0.3	2.26	NS	1	0.003	0.02	NS	1	0.9	7.90	p <0.01
Number of whorls of ray florets	2	12.8	2.08	NS	1	38.5	6.27	p <0.05	1	3.4	0.60	NS
	Source of variation: P*NSR*clippingy				Source of variation: Rep(P*NSR)				Source of variation: Clipping*Rep(P*NSR)			
	df	MS	F ratio	P value	df	MS	F ratio	P value	df	MS	F ratio	P value
Diameter of whole capitulum	2	0.9	2.04	NS	28	1.7	3.98	p <0.001	28	1.1	2.23	p <0.001
Diameter of disc florets	2	0.4	3.20	p <0.05	28	0.5	3.87	p <0.001	28	0.3	1.92	p <0.01
Number of whorls of ray florets	2	0.6	0.10	NS	28	10.7	1.74	p <0.05	28	15.0	2.47	p <0.001

Table 2.6. General Linear Model ANOVA results of main treatment effects P, NSR and P*NSR on water relations measurements.

Water Relations Measurements	Source of variation: P				Source of variation: NSR				Source of variation: P*NSR			
	df	MS	F ratio	P value	df	MS	F ratio	P value	df	MS	F ratio	P value
Solution volume (before herbivory	2	129159.3	0.10	NS	1	64560500.0	47.25	p <0.001	2	1673718.6	1.23	NS
Solution volume (after herbivory)	2	1519406.8	2.53	NS	1	928898.2	1.55	NS	2	154292.8	0.26	NS
Relative mass of blocks (before herbivory	2	3.5	0.55	NS	1	66.6	10.59	p <0.01	2	3.1	0.49	NS
Relative mass of blocks (after herbivory)	2	5.3	1.51	NS	1	43.5	12.49	p <0.01	2	0.03	0.01	NS

Table 2.7. General Linear Model ANOVA results of main treatments effects of NSR and P*NSR on plant tissue concentrations of N, P, and K.

Nutrient Analyzed	Source of variation: NSR				Source of variation: P*NSR			
	df	MS	F ratio	P value	df	MS	F ratio	P value
Nitrogen	1	8487.3	3.87	p =0.053	2	11494.6	5.25	p <0.01
Phosphorus	1	0.7	0.001	NS	2	573.3	0.79	NS
Potassium	1	46.9	0.04	NS	2	2267.5	1.84	NS

Table 2.8. Comparison of hydroponic and field grown Calendula crops.

Culture type	Culture conditions	Day of harvest	Number of leaves	Height aerial parts	Dry mass (g)			root: shoot	Author
					Aerial parts	Roots	Flowers		
Field		120	25.4 ± 3.2	46.1 ± 5.7	7.4 ± 2.1	0.9 ± 0.5	0.3 ± 0.2	0.12	Papadopoulos *et al.*, 2000
Hydroponic	floating raft 41-50mg/L P	75	12.0 ± 0.9	37.4 ± 11.1	11.5 ± 7.3	0.9 ± 0.9	1.3 ± 1.0	0.08	Papadopoulos *et al.*, 2000
Hydroponic	floating raft 100mg/L P	70*	49.4 ± 2.4	17.7 ± 0.7	2.5 ± 0.1	0.3 ± 0.02	0.9 ± 0.8	0.12	Stewart and Lovett-Doust, 2002 in review
Hydroponic	shallow culture 10mg/L P	123	170.1 ± 10.5	51.8 ± 1.3	8.4 ± 0.4	1.6 ± 0.1	0.7 ± 0.08	0.21	This study
Hydroponic	shallow culture 100mg/L P	123	219.2 ± 11.9	56.7 ± 1.2	9.4 ± 0.4	1.4 ± 0.09	1.1 ± 0.09	0.14	This study
Hydroponic	shallow culture 200mg/L P	123	220.4 ± 10.9	56.3 ± 1.2	7.9 ± 0.2	1.8 ± 0.2	1.0 ± 0.07	0.24	This study

*plants were harvested before the end of natural growth/bloom period due to plant mortality

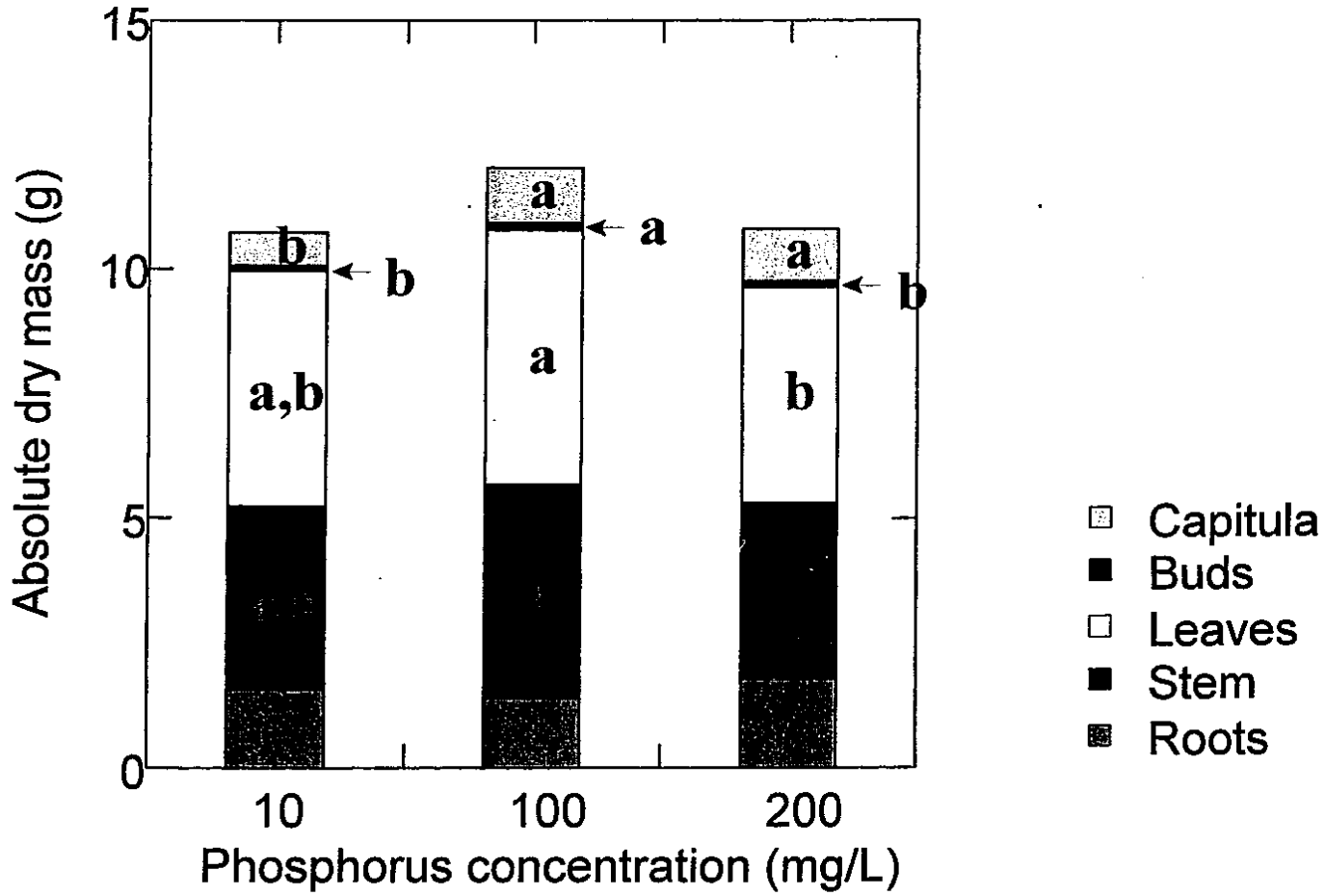

Figure 2.1. Effects of P concentration on the absolute dry mass (g) of root, stem, leaf, buds and capitula. Different letters within a tissue or beside a tissue indicate significant differences (Sheffé's post hoc comparison).

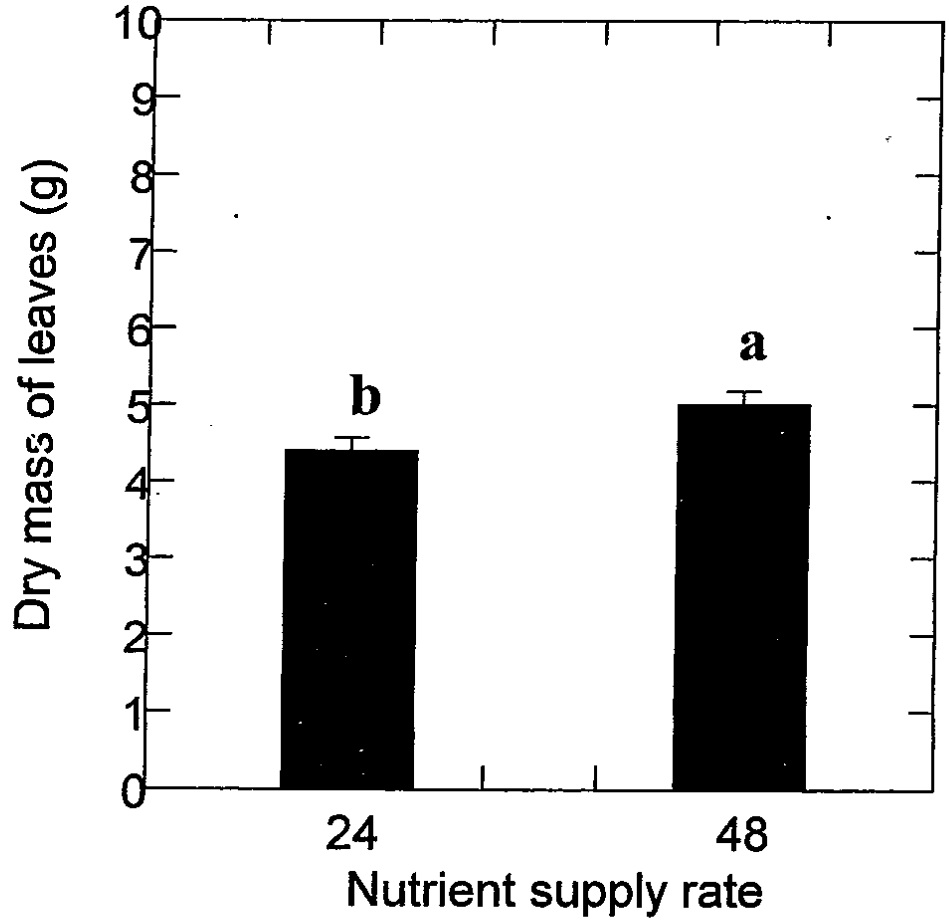

Figure 2.2 Effects of nutrient supply rate on leaf dry mass (g). Different letters indicate significant differences (Sheffé's post hoc comparison).

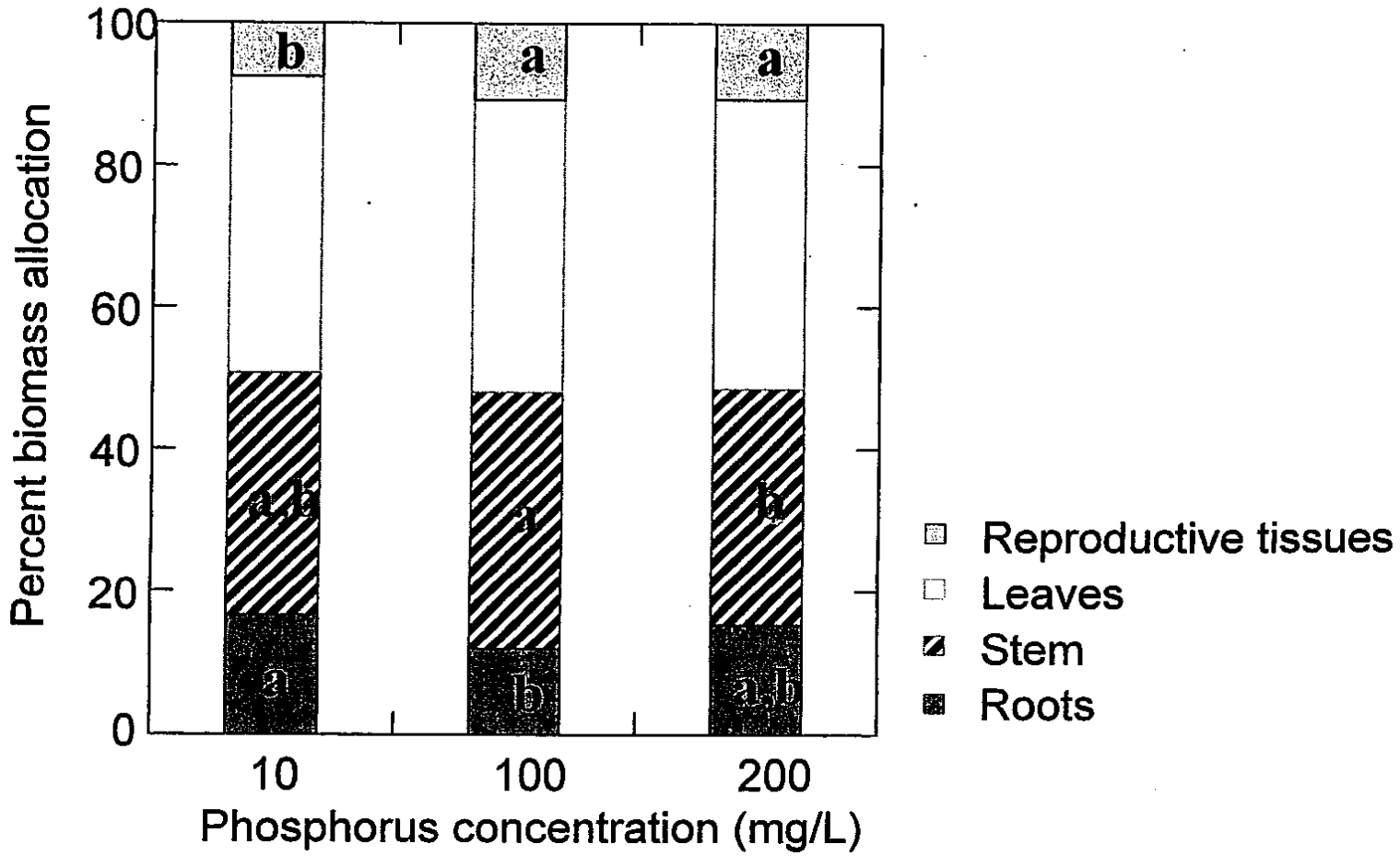

Figure 2.3. Effects of phosphorus concentration on the proportionate biomass allocated to root, stem, leaf, and reproductive tissues. Different letters within a tissue indicate significant differences (Sheffé's post hoc comparison). Leaf mass was not significantly different.

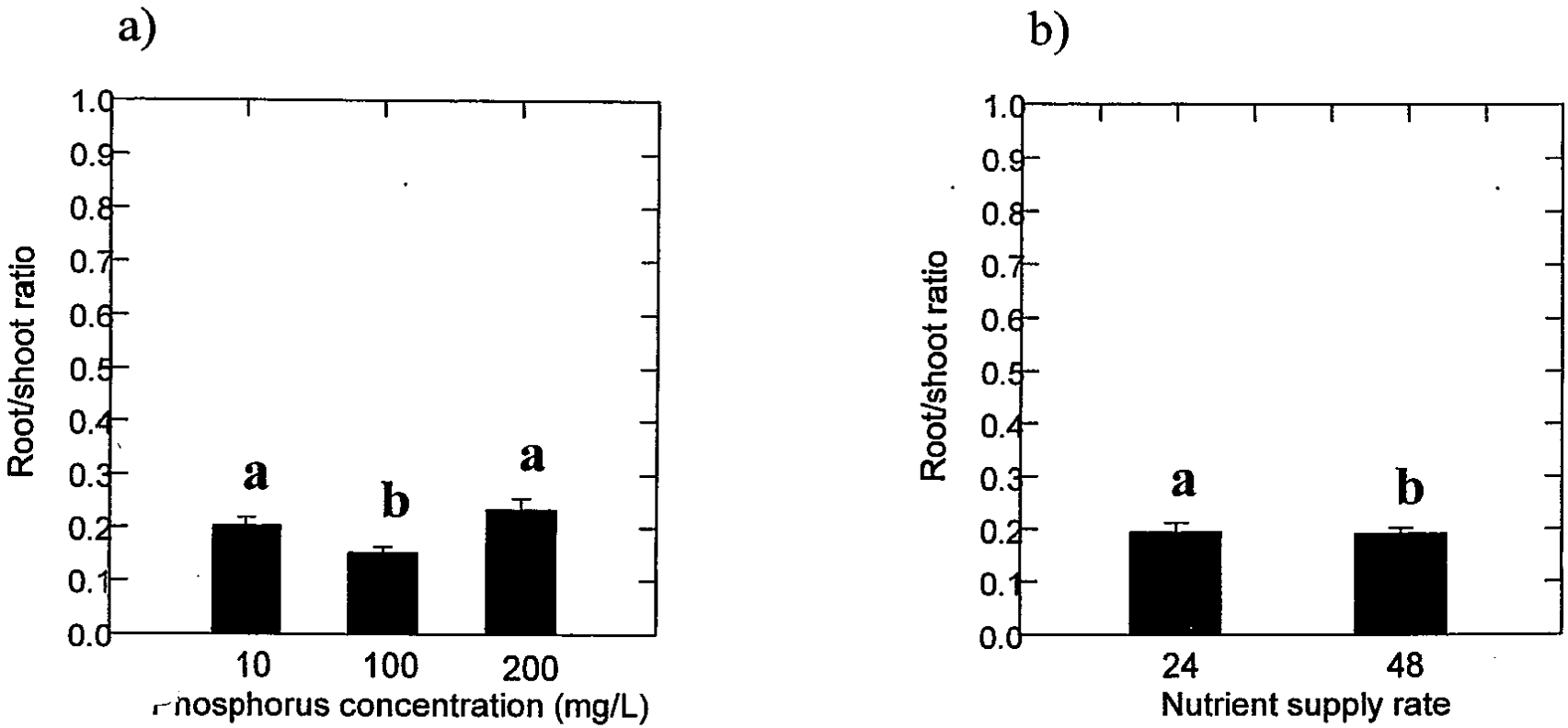

Figure 2.4. Effects on root/shoot ratio of: a) P concentration; and b) nutrient supply rate. Different letters above the bars indicate significant differences (Sheffé's post hoc comparison).

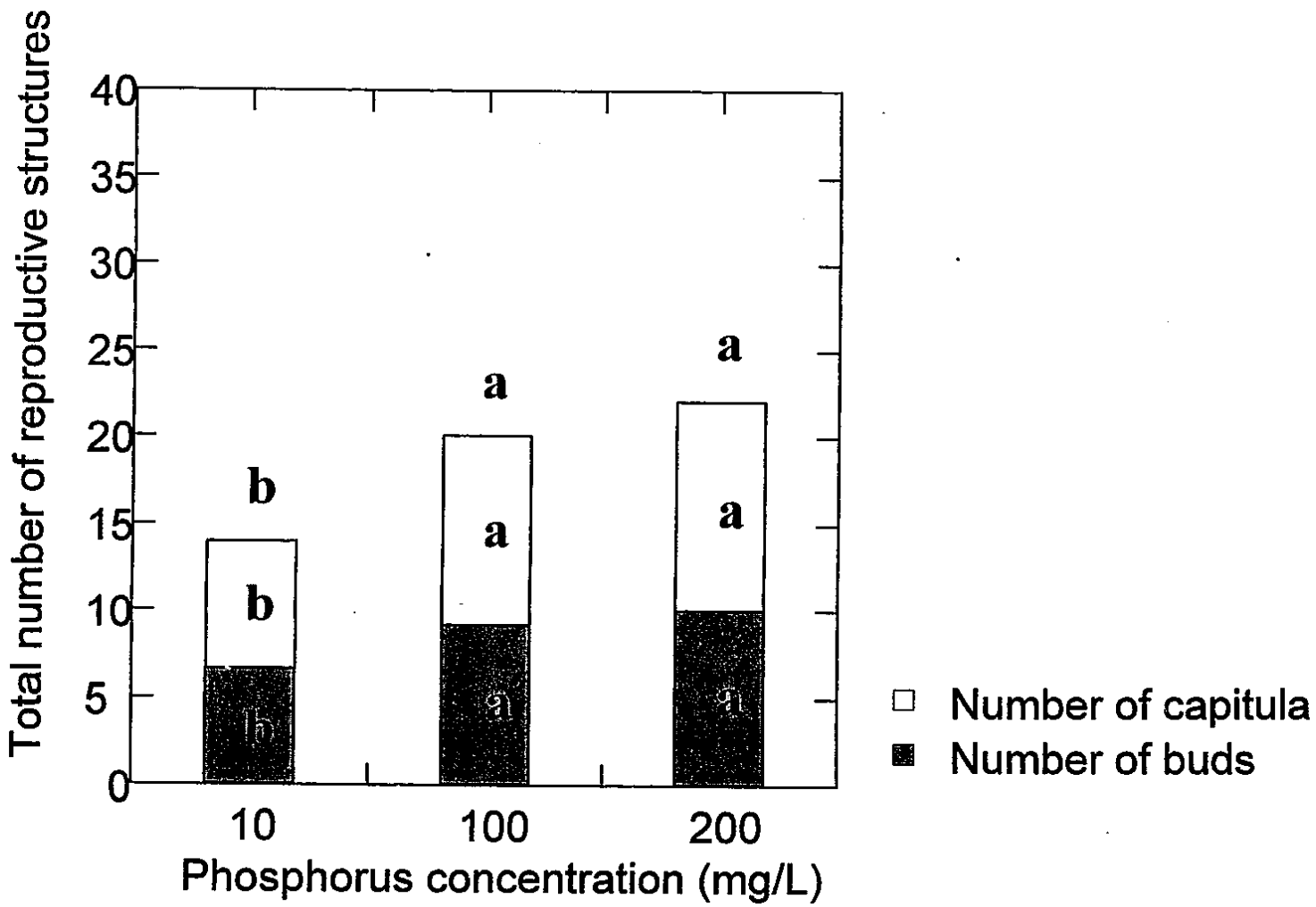

Figure 2.5. Effects of phosphorus concentration on total reproduction. Different letters within a tissue and above total number for both structures indicate significant differences (Sheffé's post hoc comparison).

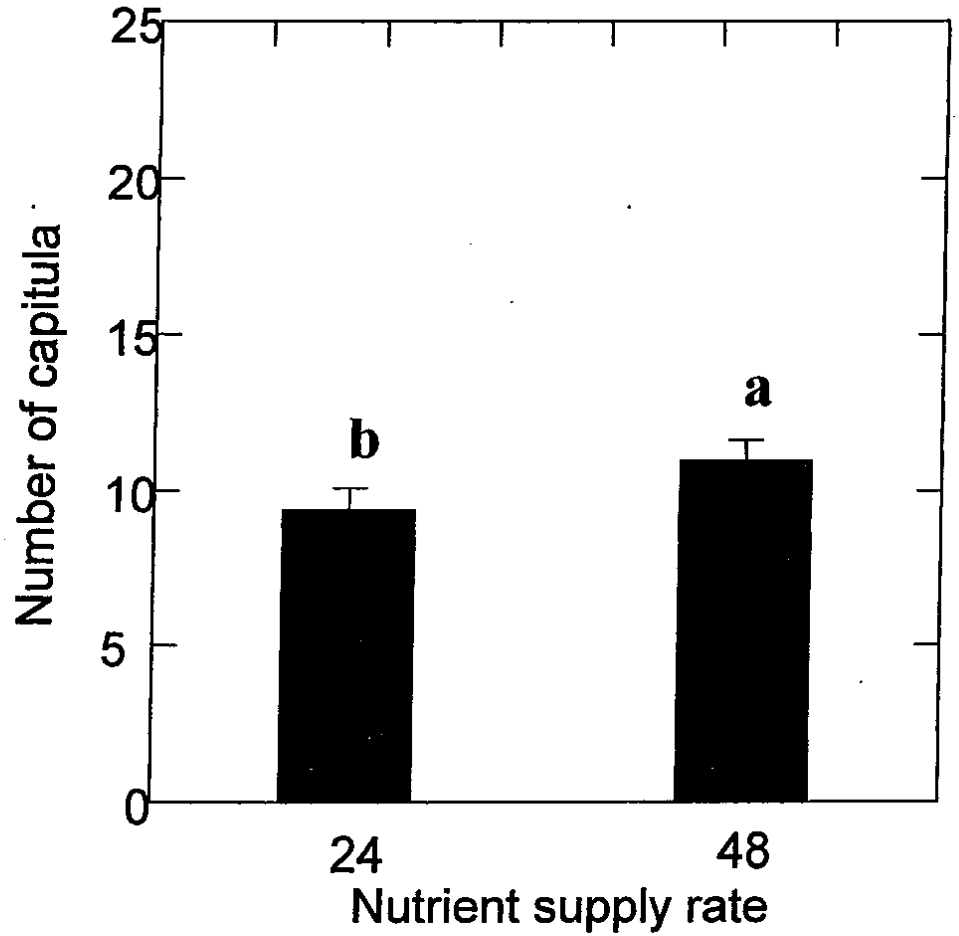

Figure 2.6. Effects of nutrient supply rate on the production of capitula. Different letters indicate significant differences (Sheffé's post hoc comparison).

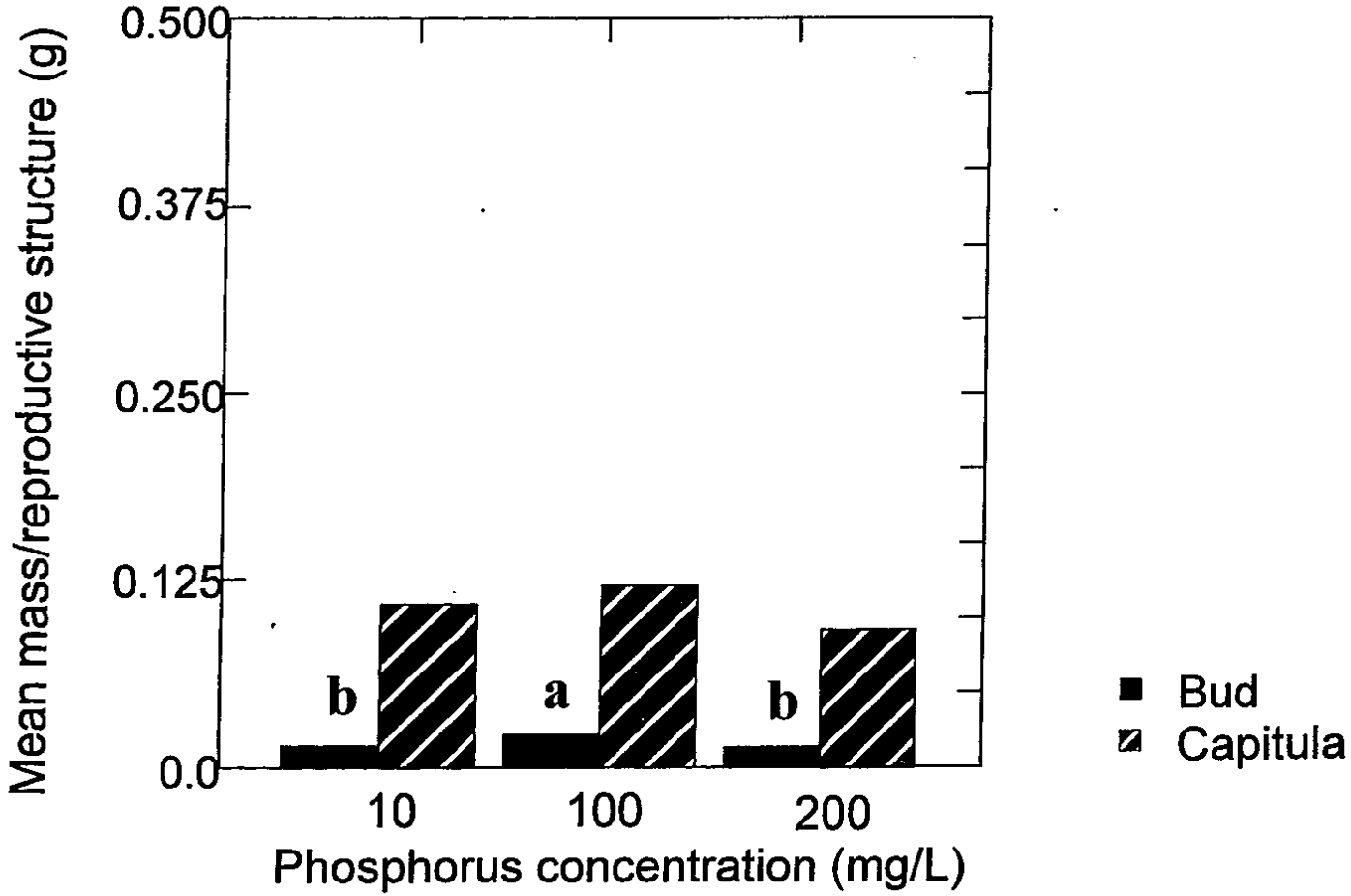

Figure 2.7. Effects of phosphorus concentration on the average mass/ reproductive structure. Different letters indicate significant differences (Sheffé's post hoc comparison). There were no significant differences for mean mass/capitula between treatments.

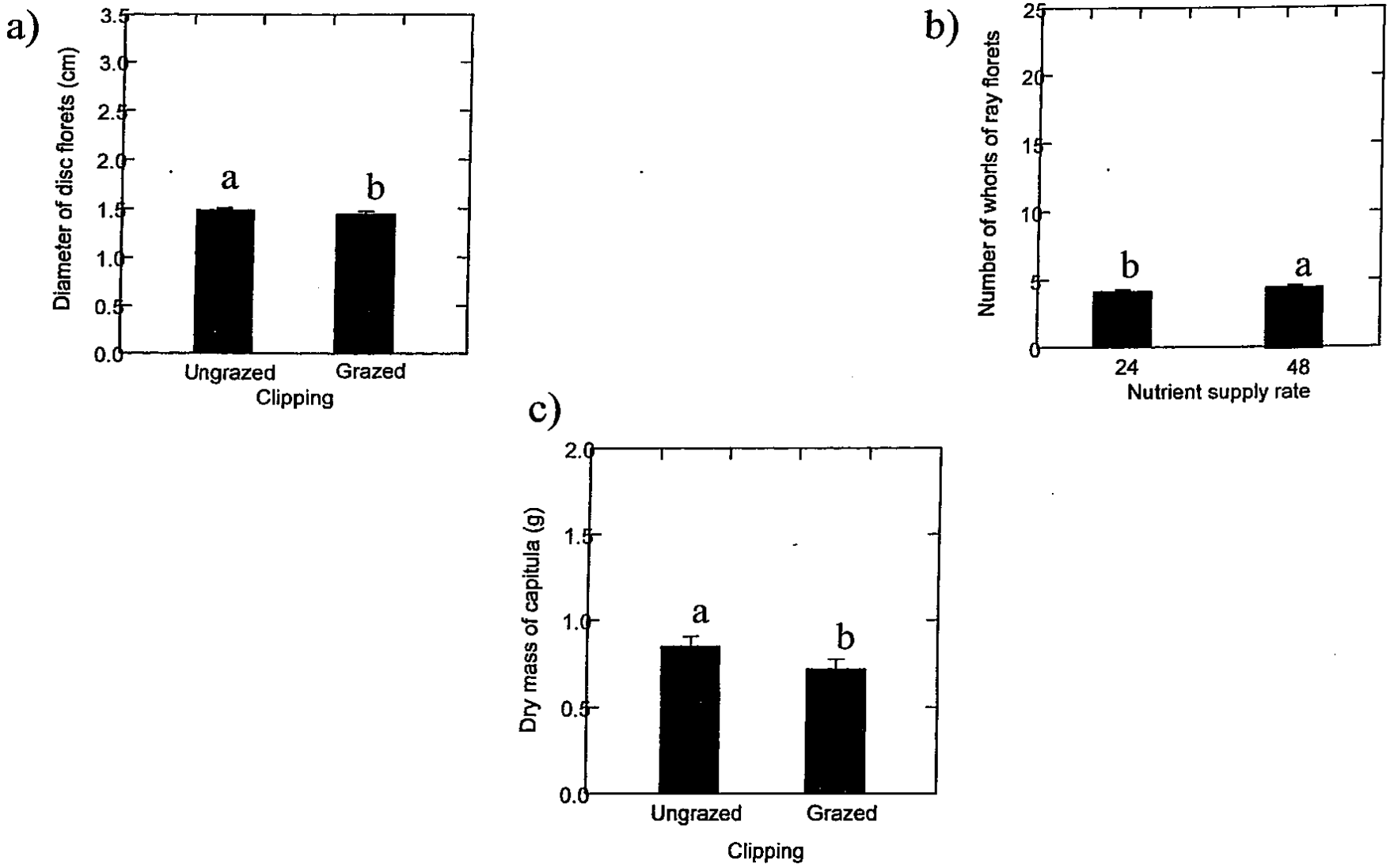

Figure 2.8. Effects of: a) clipping on the diameter of disc florets; b) nutrient supply rate on the number of whorls of ray florets; c) clipping on the dry mass of capitula . Different letters above each bar indicate significant differences (Sheffé's post hoc comparison).

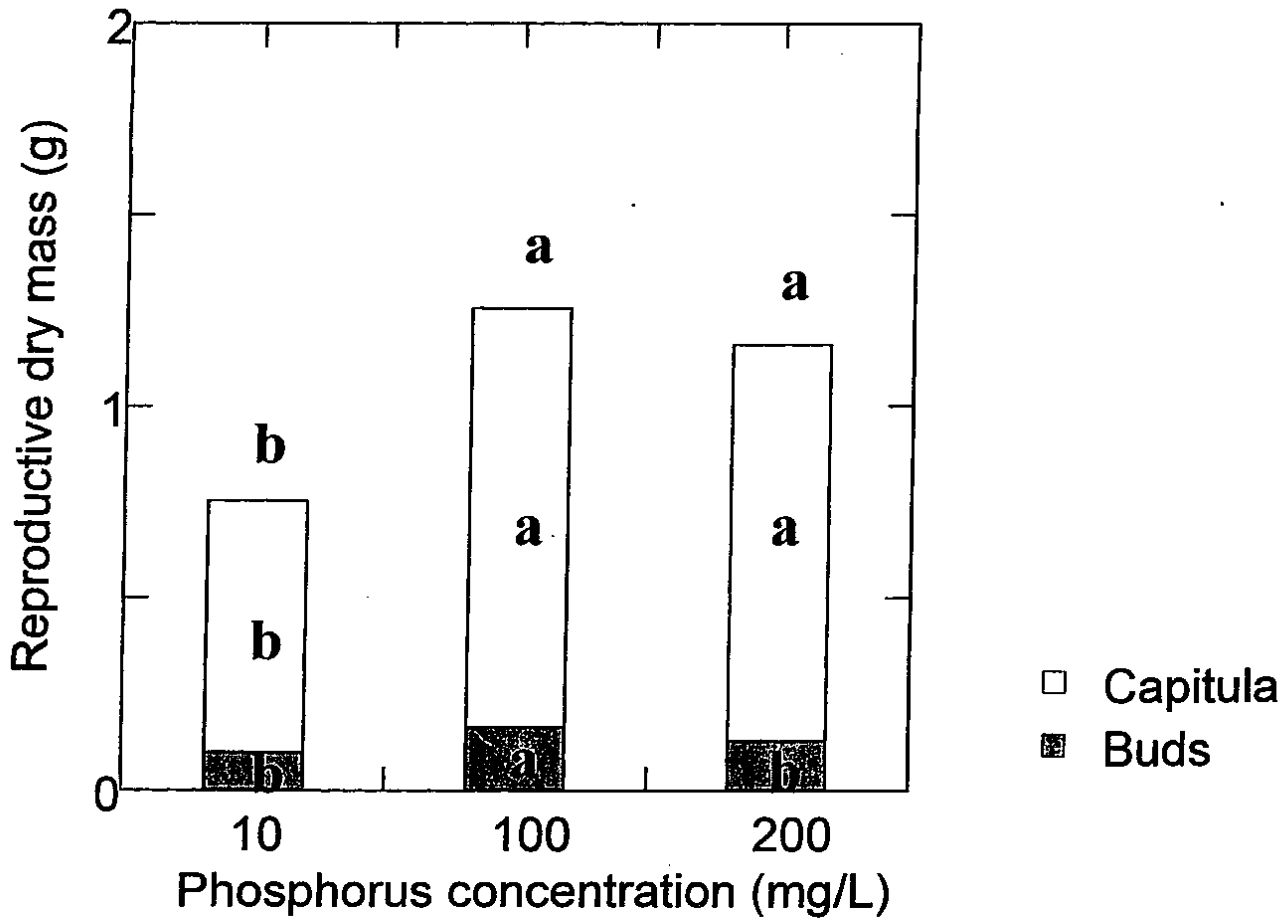

Figure 2.9. Effects of phosphorus concentration on bud and capitula dry mass. Different letters within each tissue and, for total reproductive dry mass, above each bar indicate significant differences (Sheffé's post hoc comparison).

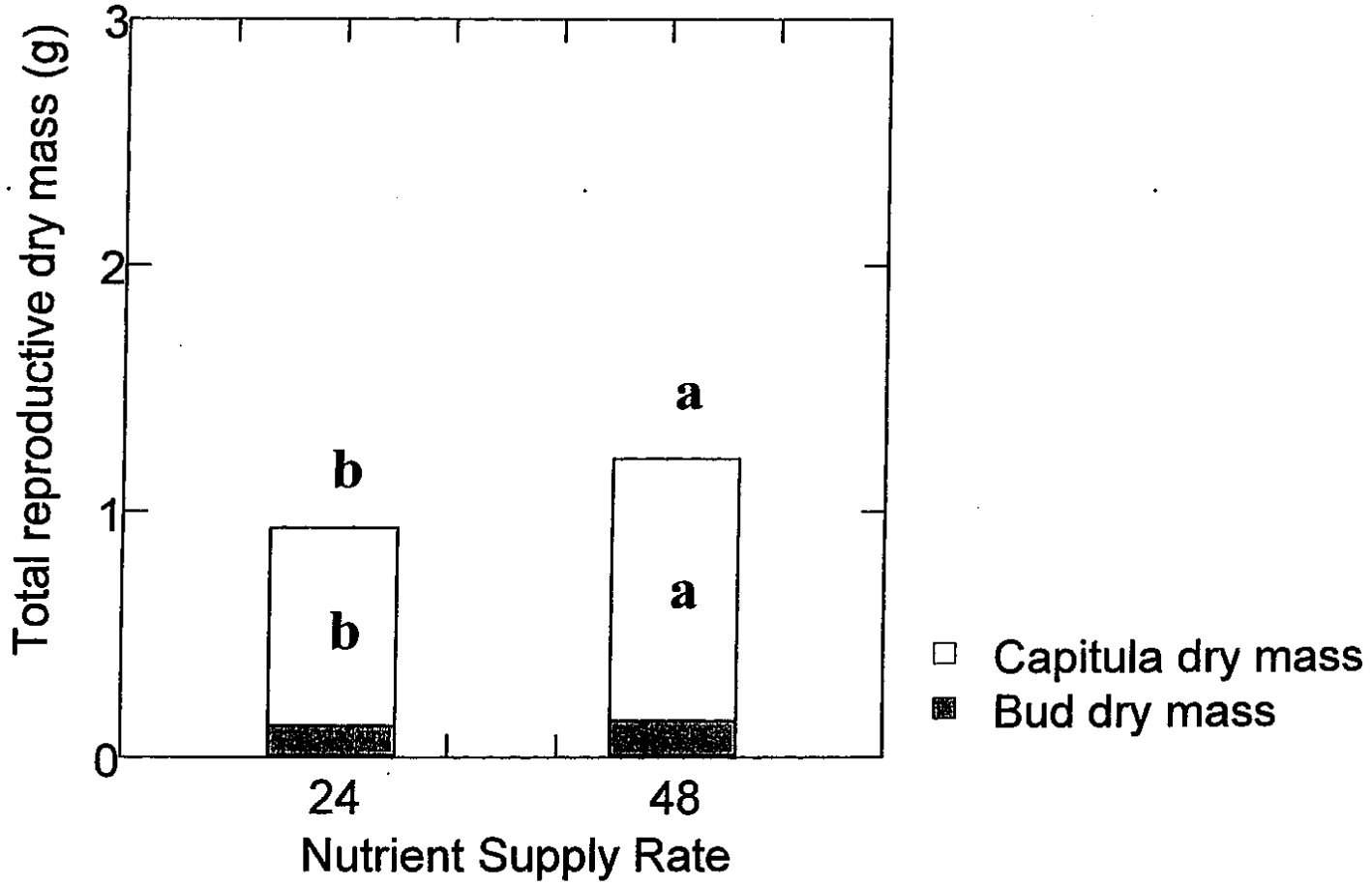

Figure 2.10. Effects of nutrient supply rate on the dry mass of buds,capitula, and total reproduction. Different letters within a tissue and above the bars indicate significant differences (Sheffé's post hoc comparison). There was no significant diffrence for bud dry mass between the two NSRs.

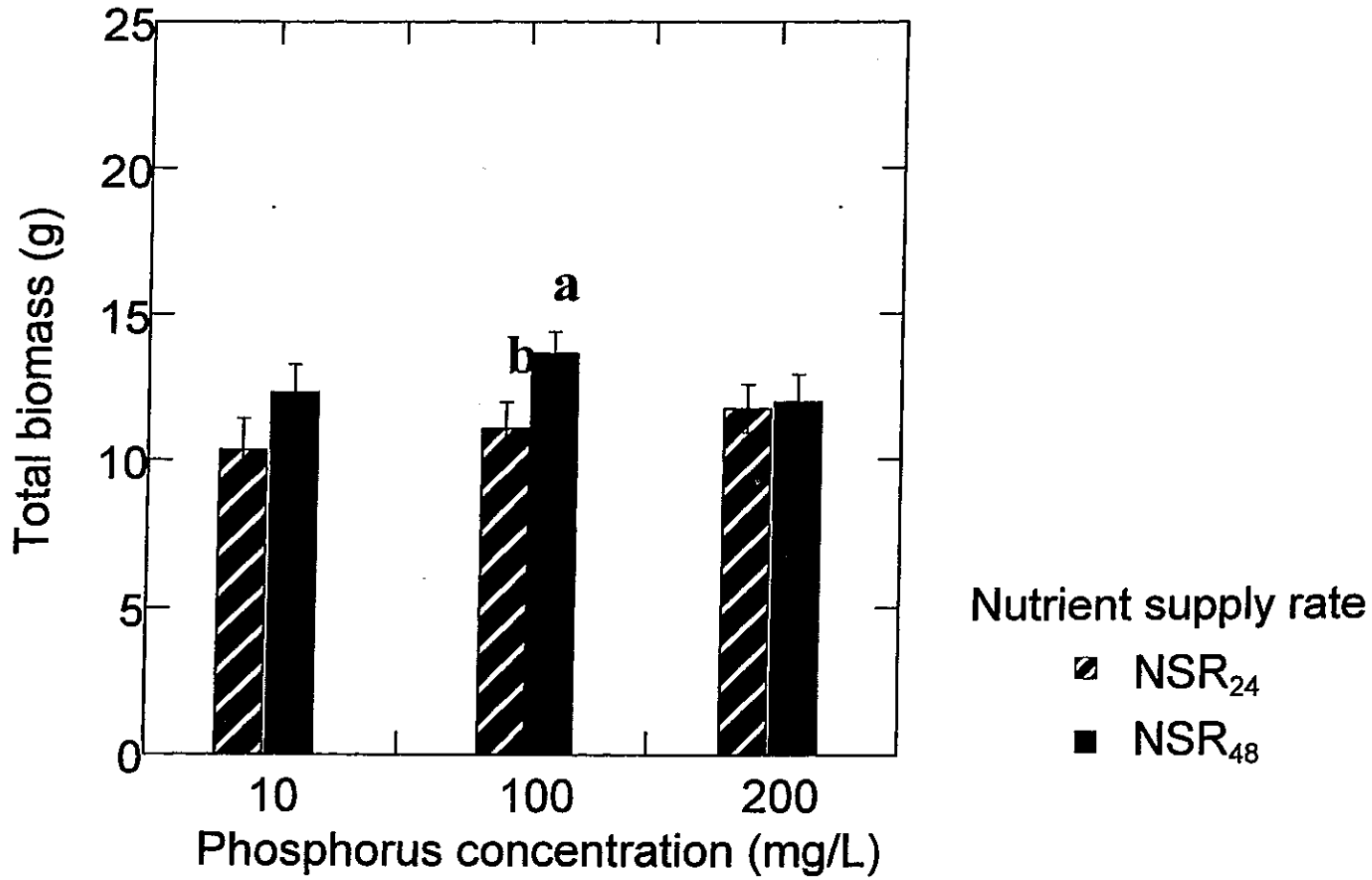

Figure 2.11. Interaction of both P concentration and NSR on the total biomass of the plant, letters indicate significant differences of the mean values between NSR's within a P concentration (Sheffé's post hoc comparison). There were no significant differences at P levels of 10 and 200 mg/L.

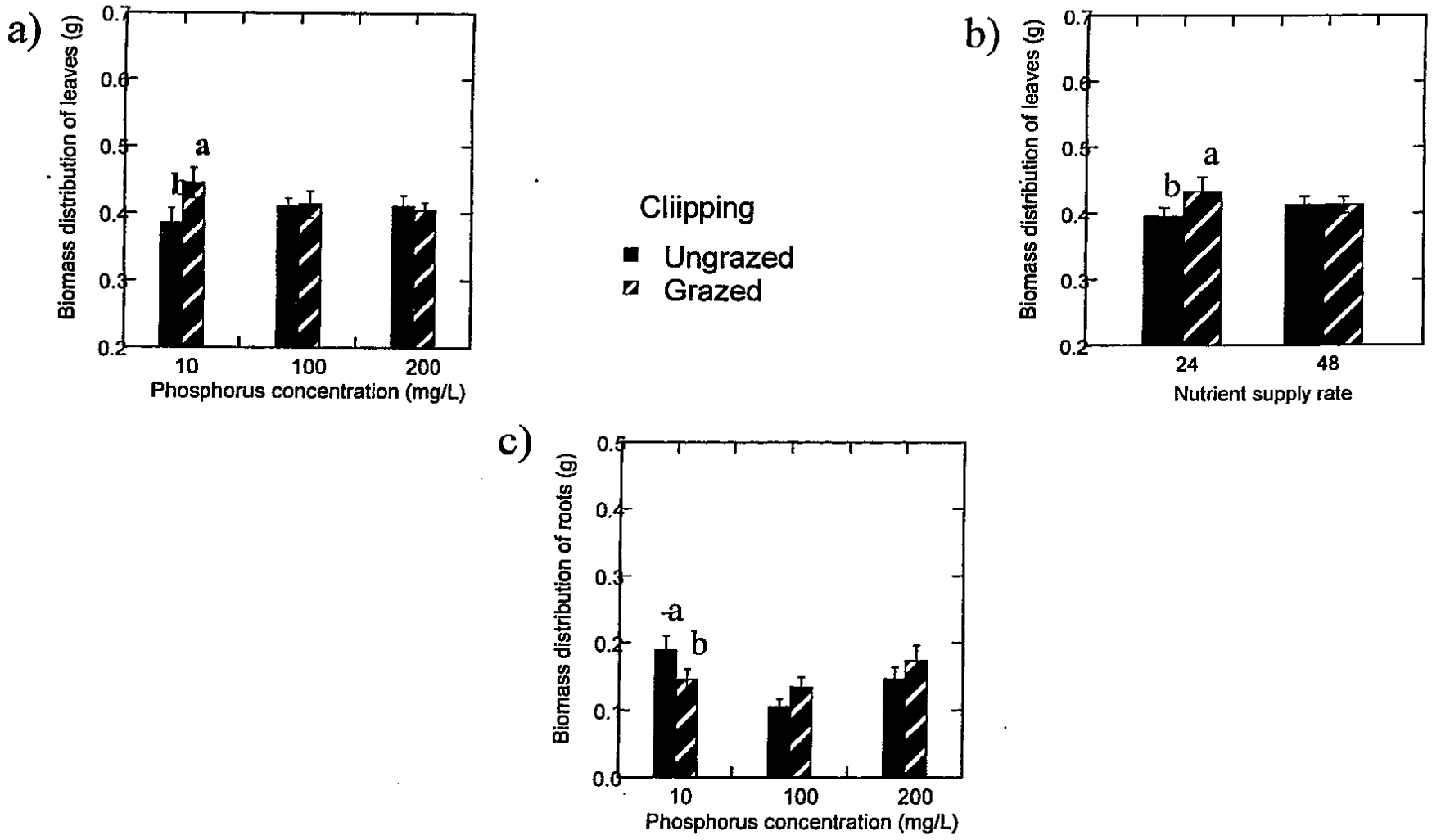

Figure 2.12. Effects of: a) P concentration and herbivory on distribution of leaf mass; b) NSR and clipping on distribution of leaf mass; c) P concentration and herbivory on the distribution of root biomass. Different letters indicate significant differences (Sheffé's post hoc comparison). Where there are no letters, differences between means were not significant.

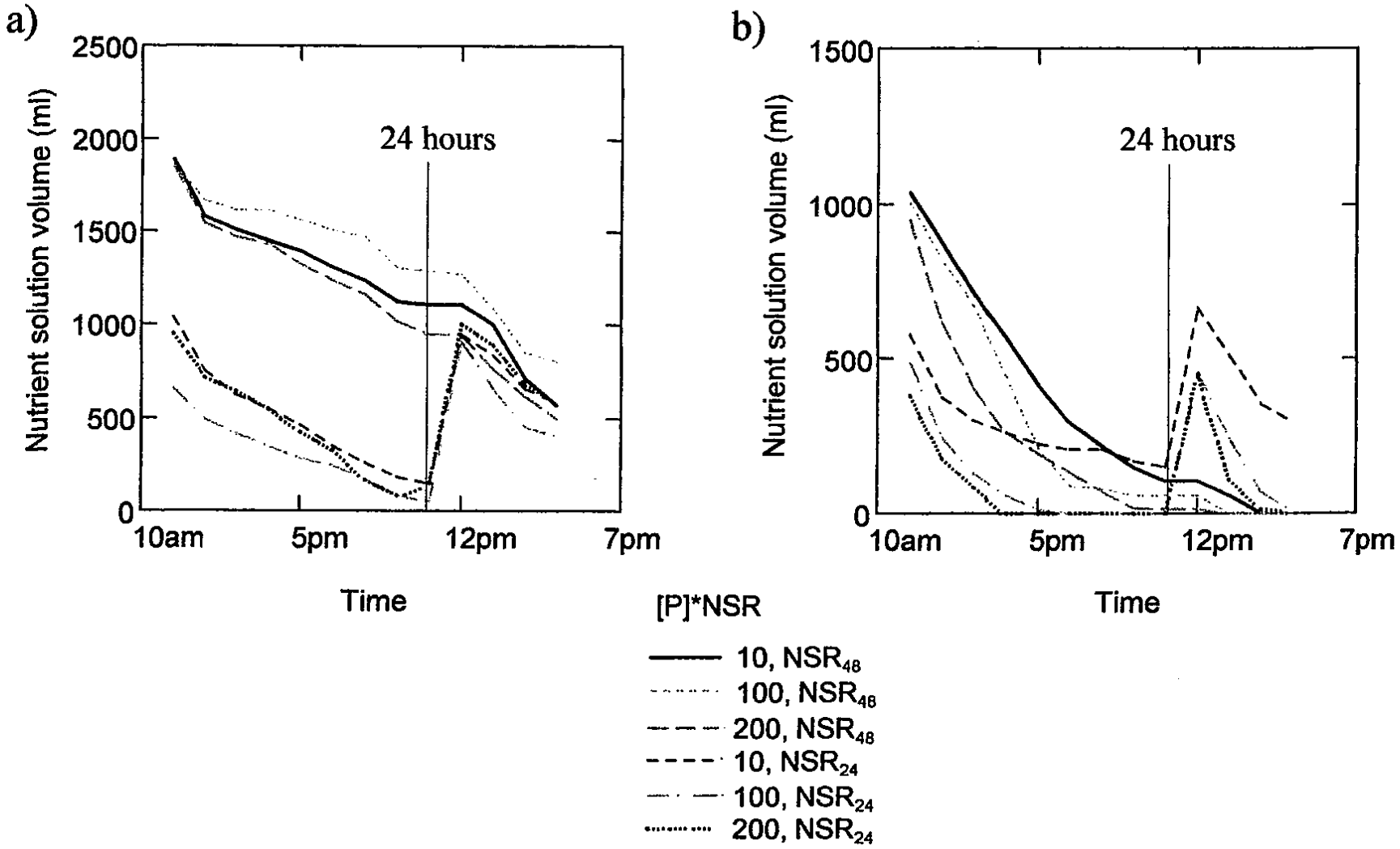

Figure 2.13. Effects of P concentration and nutrient supply rate on the changes in solution volume over time for: a) plants prior to clipping and; b) plants following clipping.

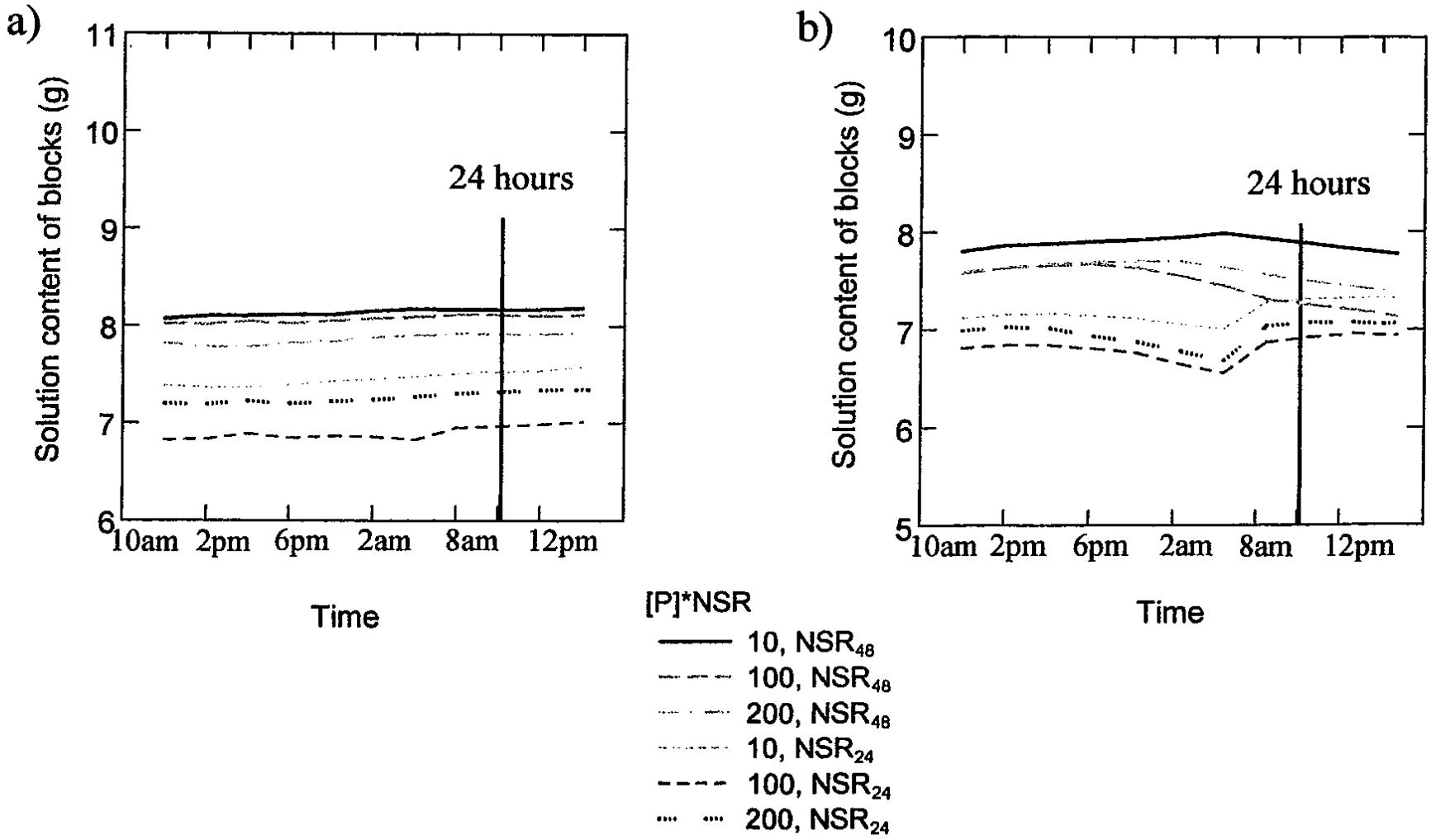

Figure 2.14. Effects of P concentration and nutrient supply rate on the changes of solution content of blocks over time on: a) plants prior to clipping and b) plants following clipping.

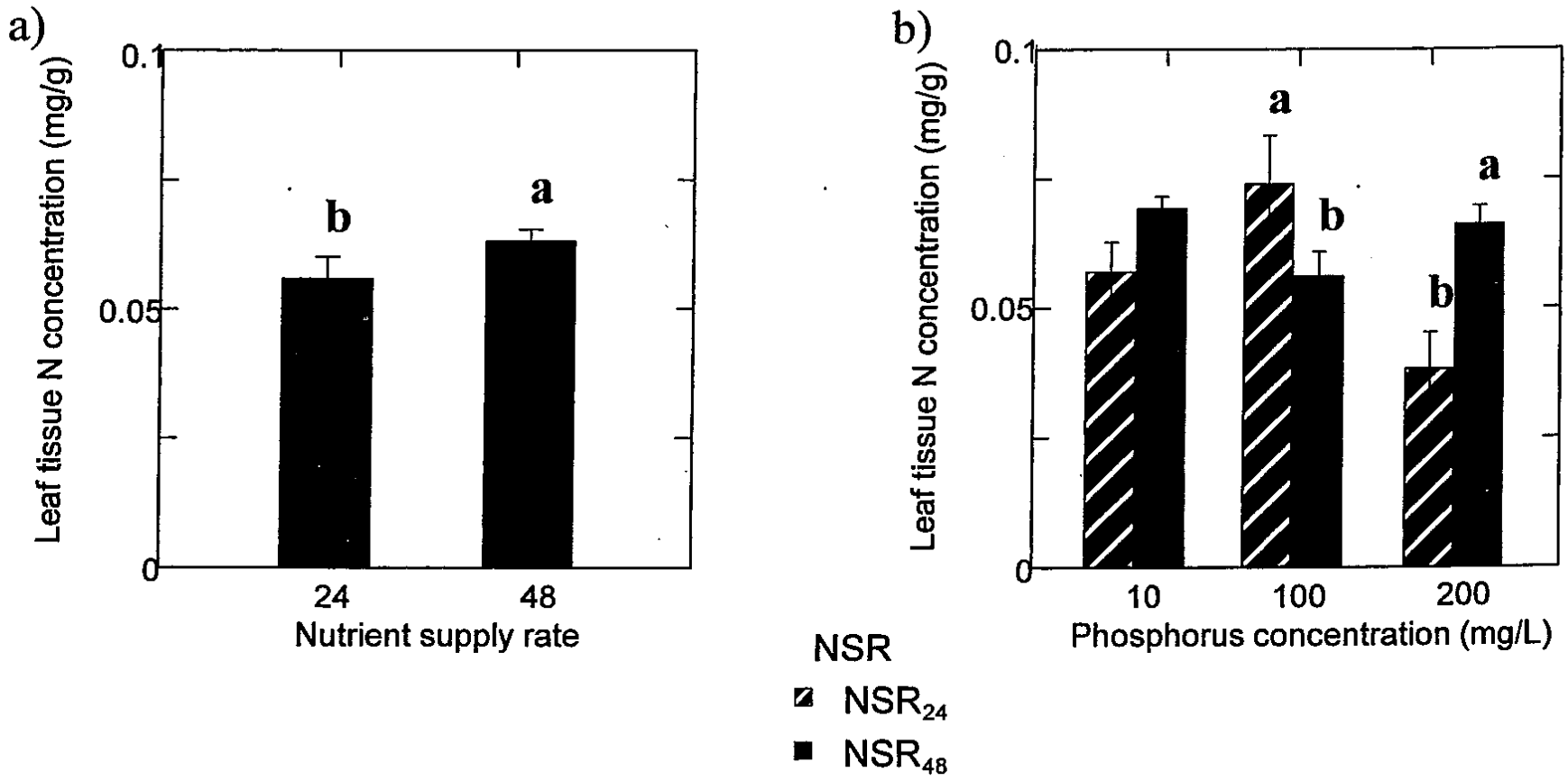

Figure 2.15. Effects of: a) nutrient supply rate on leaf tissue N concentration and; b) the interaction effects of P concentration and NSR on leaf tissue N concentration. Different letters indicate significant differences (Sheffé's post hoc comparison). Where there are no letters, differences between means were not statistically significant.

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Chapter 3

FACTORS AFFECTING FLAVONOID PRODUCTION IN THE MEDICINAL PLANT *CALENDULA OFFICINALIS* (L.)

Introduction

The modern pharmaceutical industry is based on the foundation of traditional medicinal systems that originated hundreds of years ago. In North America, at least 60 important medicines are still obtained directly from plant materials (McClatchey and Stevens, 2001). Throughout the world, the estimated sales of plant-based medicines exceeds $12 billion (Murch *et al.*, 2001), with some 7000 medicinal compounds used in Western pharmacopoeia derived from plants (Coe and Anderson, 1996). These figures do not include sales of unprescribed (over-the-counter) herbal remedies in North America. The North American herbal market is growing at a rate greater than 10% per year, with consumers spending $12 billion on natural supplements and $27 billion annually on herbal remedies and alternative therapies in 1997 (Greenwald, 1998).

Herbal remedies contain bioactive plant molecules, which are described as secondary metabolites because they have no known function in the primary plant metabolism, creating new tissue or producing energy. However, these bioactive molecules may provide plants with defense mechanisms against herbivory (Janzen, 1974; Harborne and Williams, 2000), disease (Cowan, 1999; Paiva, 2000), competition (Basile, 2000; Briskin, 2000), or even environmental stress (El-Demerdash, 2001; Warren *et al.*, 2002), and may affect the growth and development of other organisms (Seigler, 1996; Walling, 2000; Karban and Maron, 2002).

A plant's response to environmental challenges activates signal transduction pathways that initiate particular defense responses (Walling, 2000). Chrispeels and

Sadava (1994) characterized the production of secondary metabolites against invaders as either constitutive or inducible. Constitutive systems are those which are present in a plant whether the plant is attacked, or not (Walling, 2000). Examples of constitutive systems are the terpenoids, alkaloids and phenolics (Chrispeels and Sadava, 1994). Inducible systems include those which are not present in the plant prior to invasion or attack by a predator, bacterium, virus or fungus, but rather are induced, at the time of invasion/attack (Chrispeels and Sadava, 1994) or they may be present at a lower level, which increases following attack (Litvak and Monson, 1998).

As a defense mechanism against herbivores, buildup of secondary metabolites in plant tissues can reduce palatability and decrease digestibility for herbivores (McNaughton, 1983); it could also cause physical injury to the herbivore (Freeland and Janzen, 1974), discouraging further attack and damage. Collantes *et al.* (1997) and Gianoli and Niemeyer (1998) found an induced increase in the concentration of hydroxyamic acids in experiments using rye (*Secale cereale*), which was exposed to simulated defoliation, and wild wheat (*Triticum uniaristatum*), which was grazed by aphids. Likewise, Litvak and Monson (1998) observed a significant increase in the production of monoterpenes in several conifer species which were treated with simulated herbivory, and an even greater response in ponderosa pine (*Pinus ponderosa*) experiencing natural herbivory by tiger moth larvae (*Halisdota ingens* Hy. Edwards: Lepidoptera). These increases in monoterpenes occurred beyond the constitutive levels that are naturally present in the tree species. Feller (1995) observed an inverse relationship between concentrations of phenolic compounds in Dwarf Red Mangrove (*Rhizophora mangle*), growing in a nutrient-limited mangroves in Belize, and levels of

herbivory by specialist herbivores, such that increased phenolic levels in leaf tissue were associated with a decreased rate of herbivory.

Besides herbivores, other plant pests and pathogens that may induce a plant to produce chemical defenses are pathogenic bacteria, fungi, and viruses. When these organisms infect a plant, the cells adjacent to the site of infection often release chemicals called phytoalexins, which may be either phenolics or terpenes depending on the species (Chrispeels and Sadava, 1994) These may kill the invader, as well as local cells in the area (Paiva, 2000). Continued plant response to invasion includes the deposition of lignin in the cell walls (Rayachherty *et al.*, 1996) which strengthens them; and the secretion of enzymes and/or proteins into intercellular spaces and vacuoles (Pearce, 1996), which inhibit the growth of pathogens in various ways (Chrispeels and Sadava, 1994). Secondary metabolites appear to exert their effects on pathogens primarily by enzyme inhibition or enzyme destruction, within a particular metabolic pathway of the pathogen (Kalvatchev *et al.*, 1997; Alcaráz *et al.*, 2000; Stermitz *et al.*, 2000).

Inter-plant competition may also stimulate secondary chemical defenses in plants (Siegler, 1996). This chemical interaction between plants by modulation of another plant's development is known as allelopathy (Basile *et al.*, 2000). Allelopathy may function as an adaptive response for some plants to outcompete their neighbours in order to enhance their successful survival and/or reproduction. Basile *et al.* (2000) studied the allelopathic activity of extracts from *Castanea sativa* leaves, as well as the application of quercetin, rutin and apigenin (isolated phenolics) on seed germination in *Raphanus sativus*. All of these compounds caused a significant decrease in seed germination and diminished root and epicotyl growth in *Raphanus sativus*. Hypothetically, in nutrient-limited environments, competition should be greater for whatever nutrients are available

(Chapin, 1980). As a result, plants would be more likely to have allelopathic activity if allelopathy were an adaptive response. Allelopathic effects may be exerted between species or between individuals of the same species, particularly when lack of moisture or nutrients is limiting growth (Harborne, 1988). Plant-to-plant interference is a complex combination of competitive interference for resources and allelopathic chemical reactions (Fuerst and Putnam, 1983).

Increased secondary metabolite production in plant tissues may also be an adaptation to environmental stress for stress-tolerant plants; with a stress being defined as any condition that restricts production (Grime, 1977) and especially in resource-limited growth conditions, leading to low palatability (Grime, 1977; Chapin, 1980; McNaughton, 1983; Coley *et al.*, 1985; Bazzaz, 1987). Resource availability has been proposed as the major determinant of both the extent and type of plant defense (Coley *et al.*, 1985). The evolutionary response of plants growing in resource-limited conditions is a slow growth rate, in comparison to the faster growth rate of species in more nutrient rich conditions (Chapin, 1980), with slow growth rates favouring greater protection (larger investments in anti-herbivore defenses, Coley *et al.*, 1985).

Two schools of thought exist as to why slower growth rates may encourage anti-herbivore defenses have emerged; these hypotheses may not be mutually exclusive. The first follows upon the premise that slow-growing plants are longer-lived, as are their individual plant parts (McNaughton, 1983), especially the leaves, which contain a large proportion of the nutrient pool (Chapin, 1980). In this case, since, in nutrient-limited soils, new growth to replace the lost photosynthetic tissues is slow, the loss of leaves and nutrient pools with them is costly; hence benefit is gained by producing high concentrations of 'defensive' compounds to protect the plant tissues against grazing

pressure (Chapin, 1980). The second explanation addresses the limitation of resources within the plant, due to a nutrient-limited environment, specifically major growth-limiting minerals nitrogen and phosphorus. Adaptation to nutrient-limited environments results in a reduction in plant growth rate, compared to species in nutrient-rich environments (Gerloff, 1963). Slower rates of growth can be supported by nutrient-limited environments because nutrients needed for plant growth are not available; therefore slow-growing plants put less demand on nutrients for uptake (Gerloff, 1963). The nutrient-limited environments of slow growing species are typically deficient in either nitrogen or phosphorus supply, or both (Beadle, 1962). Both phosphorus and nitrogen are needed for assimilation in structural growth and when limited in the environment cause a build-up of non-structural carbon, typically in the leaves, in the form of carbohydrates, often starch (Ericsson, 1995). These excess non-structural carbon skeletons are then used to produce (synthetically) expensive carbon-based defense chemicals, such as phenols (McNaughton, 1983). If under in these conditions, nutrients become no longer limiting, then the carbon in defense chemicals could be used for plant structures in growth. Although this second explanation is not a direct or preventative response to herbivory, it, too, would predict a lower rate of herbivory in nutrient-stressed plants than in plants grown in nutrient-limited resources and an inverse association between growth rate and concentration of secondary chemicals. This second interpretation is the basis of Bryant *et al.*'s (1983) "carbon/nutrient balance" hypothesis, which views synthesis of secondary compounds as an adaptive response to herbivory that evolved under constraints of resource limitation (Feller, 1995).

Globally, Certain regions are known to be more nutrient deficient than others on the basis of soil mineral composition and climate, which often dictates the types of plants

that will, survive and adapt (e.g., tropical, sub-tropical, arid or semi-arid, and boreal regions). Mediterranean climates, which are mainly phosphorus- and somewhat nitrogen-deficient (FAO, 2000), are characterized by winter rains (three times summer precipitation) and warm dry summer months with moisture deficits (Yaalon, 1997). This type of climate is described as having a xeric moisture regime and the many plants adapted to this climate are known very broadly as xerophytes (Beadle, 1962), which are also sclerophyllous in nature (Loveless, 1961). Sclerophylly is hardening of leaf tissues through the thickening of the leaf cuticle and outer epidermal wall, and sclerification particularly of the vascular bundle sheaths and leaf margins (Turner, 1994), which is suggested as a result of limited nutrients (specifically phosphorus) (Loveless, 1961) and/or an adaptive protective response to dry habitat (Beadle, 1968), such as those found in the Mediterranean climate. The xeric moisture regime of Mediterranean climates exists on all continents, typically on the western parts between the cooler temperate zone and hot dry desert zone (Yaalon, 1997). Along with being P- and N-deficient, soils of these regions are also often very calcareous and alkaline (Henkin *et al.*, 1996; Cocks and Osman, 1996; Carreira *et al.* 1997; Yaalon, 1997). Since it has been found that species growing in nutrient-limited environments produce greater amounts of secondary metabolites (McNaughton, 1983), and Mediterranean habitats are nutrient-limited, then we may speculate that xerophytic species grown in these climates will produce greater amounts of secondary metabolites than plants grown in nutrient-rich habitats. Janzen (1974) suggested that the chemical characteristics of plants native to resource-limited habitats may also be an adaptive mechanism of defense against herbivores. He reasoned that the loss of leaves to herbivores would have a greater impact on a plant growing on infertile soils than on fertile soils.

The uniform conditions provided by greenhouse hydroponics should minimize plant-to-plant variation in secondary metabolite production, while investigating metabolite production for improvement of medicinal crops. Applying various stresses to plants can activate inducible pathways that enhance production of secondary metabolites (Harborne, 1988). Various stressors can be deliberately manipulated in a controlled greenhouse experiment to achieve this effect, such as nutrient availability, nutrient supply rate and the simulation of herbivory. The availability of nutrients has been shown to affect the allocation of carbon to carbon-based plant defenses, such as phenolic compounds (McNaughton, 1983). Increased concentration of specific nutrients, such as phosphorus, effectively increased the concentration of phenolic compounds found in plant tissues as does herbivory (Feller, 1995) since an array of phenolics have been identified as a plant defense against herbivores (Chrispeels and Sadava, 1994). The availability of nutrient resources, in terms of both concentration and supply rate, influences the quantity and quality of secondary metabolites (Bryant *et al.*, 1983; Coley *et al.*, 1985). Coley *et al.* (1985) suggested that slow-growing plant species, in nutrient-limited environments produce more secondary metabolites, particularly carbon-based defensive compounds, than fast-growing species in nutrient-rich environments, and are therefore less palatable to herbivores. Along with manipulating the availability of nutrients in concentration, the supply rate of nutrients can also be manipulated to affect the production of secondary metabolites. In the present study, *Calendula officinalis* is a slow-growing species (Breemhar and Bouman, 1995), native to the Mediterranean basin (Duval, 1993). This makes *C. officinalis* an attractive medicinal plant for investigation of cultivation practices to maximize metabolite output and consistency.

There are three major classes of secondary plant compounds involved in plant-animal interactions: nitrogen compounds, terpenoids, and phenolics (Harborne, 1982). There are many different subclasses within each of these classes (Table 3.1). Two basic pathways biosynthesize plant phenolics: the shikimic acid pathway and the malonic acid pathway (Taiz and Zieger, 1998). The shikimic acid pathway synthesizes most plant phenolics by conversion of simple carbohydrate precursors from glycolysis and the pentose phosphate pathway (Taiz and Zieger, 1998). In this process, the shikimic acid pathway gives rise to the amino acids phenylalanine, tyrosine, and tryptophan (Paiva, 2000). Phenylalanine ammonia lyase (PAL) converts phenylalanine to cinnamic acid (cinnamate) which is the precursor to thousands of phenolic compounds, including simple phenolics, phenylpropanoids and flavonoids (Paiva, 2000). Some studies have shown that, in several different plants species, the activity of PAL can be increased by environmental factors, such as nutrient limitation, light, and fungal infection (Hahlbrock and Scheel, 1989). Phenols have been used for many years, in different forms for their antimicrobial effects in soaps and antiseptic spray, such as throat sprays, and disinfectant solutions (Tortora *et al.*, 1998). Several studies have also investigated effects of flavonoids, a specific group of phenolics, on microbes. Both Sato *et al.* (2000) and Alcaráz *et al.* (2000) studied the effects of flavonoids against methicillin-resistant *Staphylococcus aureus* (MRSA). Sato *et al.* used a crude extract of *Scutellaria barbata*, an herb used in traditional Chinese medicine, and determined a selective toxicity of this extract to *S. aureus* and MRSA. Alcaráz *et al.* also found significant inhibition of MRSA with synthetic flavonoids, chalcone, 2′(OH)-chalcone, 2′4′$(OH)_2$-chalcone and 2′4$(OH)_2$-chalcone. Several other investigations have reported a significant positive inhibition of

bacterial cultures when treated with varying flavonoids from different plants (Báez *et al.*, 1998; Basile *et al.*, 2000; Hernández *et al.*, 2000).

Flavonoids are a subcategory of the phenolic class of secondary plant compounds, which are carbon-based compounds, only having the elements C, H and O. There are approximately 4000 different structures of flavonoids and they are found universally in angiosperms, gymnosperms and ferns (Hollman *et al.*, 1996). The flavonoid skeleton is made up of a three ring molecule, two of the rings are aromatic, and connected by a heterocyclic central ring (Paiva, 2000) (Fig 3.1). In plant ecophysiology, flavonoids have been found to have anti-herbivore, anti-pathogenic, and alleopathic effects, and increase in concentration in response to environmental stress (Harborne and Williams, 2000). Their biological activity depends on their chemical structure and the relative orientation of various moieties on the molecule (Taig and Zeiger, 1998). Flavonoids have also been noted for their antioxidant/free radical scavenging effects, which can protect cells from detrimental effects of aging (Karakaya and Nehir EL, 1999; Naguib, 2000), cancer (Ito *et al.*, 1999; Yang, 2000; Marchand, 2002), and athersclerosis (Cook and Samman, 1996; Sanchez-Moreno *et al.*, 2000). Sanchez-Moreno *et al.* (2000) investigated the antioxidant efficiency of a group of 11 polyphenols (a group of phenolic compounds) and 2 vitamins by measuring their ability to inhibit low-density lipoproteins, which are important in the pathogenesis of atherosclerosis. They found that the polyphenols showed dose-dependent inhibition of low-density lipid oxidation, greater than the common antioxidant vitamins C and E. A supporting investigation by Naguib (2000) concerning the radical-scavenging effects of some antioxidants found quercetin and rutin to have 10 times the antioxidant activity of vitamin C. Harborne and Williams (2000) reviewed many studies that found flavonoids to have anti-inflammatory activity by the

inhibition of cyclo-oxygenase and/or the 5-lipoxygenase pathways of arachidonate metabolism. Bezaková *et al.* (1996) found isorhamnetin glycosides (a group of flavonols) isolated from *C. officinalis* flowers to have inhibitory activity on lipoxygenase taken from rat lung.

In this study I attempted to identify the growing conditions that could increase the production of four of the predominant flavonoids found in *C. officinalis*: rutin, quercetin, isorhamnetin-3-O-glucoside (a methylated derivative of quercetin), and isorhamnetin-3-rutinoside (narcissin) (Fig. 3.2a, b, c, d). We also sought to reduce plant-to-plant variability in terms of concentration of these compounds.

Materials and Methods

Experimental Design

Plants were exposed to a factorial combination of treatments by varying phosphorus concentration, nutrient supply rate, and applying simulated foliar herbivory performed as a clipping treatment or leaving plants intact (control). The phosphorus concentrations used were 10mg/L (baseline concentration), 100mg/L and 200mg/L. Each concentration was applied to 12 tubs, each tub containing 11 plants. All other essential macro- and micro-nutrients needed for plant growth remained the same across all treatments for the duration of the experiment. Two nutrient supply-rate regimes were used with plants being provided nutrients either one pulse of nutrients/24 hours (NSR_{24}) or one pulse of nutrients/48 hours (NSR_{48}). Six tubs of each phosphorus concentration were supplied with NSR_{24} and six with NSR_{48}. Thus over a 48 hour period all plants received the same total amount of nutrient solution. Foliar herbivory was simulated by

clipping 50% of the leaf tissue for each leaf present on Day 54. Of the 10 plants in each tub that were treated with a combination of one concentration of P and one nutrient supply rate, five of them were exposed to simulated grazing and five remained ungrazed. Flowers were dried to a constant mass in a drying oven at 32°C. Results in terms of plant growth and capitulum productions are reported elsewhere (Chapter 2). The present study explores the concentrations of four flavonoids in capitula harvested from each treatment.

Extraction of flavonoids compounds

The dried, harvested flowers from each plant in a given treatment were pooled and ground in liquid nitrogen. Approximately 0.1g of the ground material was placed in a labeled screw-cap centrifuge tube. Ten millilitres of 98% high pressure liquid chromatography (HPLC) grade methanol was added for the extraction. This mixture was then vortexed with a Vortex-Genie (Fisher Scientific, Nepean, ON, Canada), sonicated using a Sonicor ultrasonic bath (Sonicor Instrument Corporation, Copiague, NY, USA) for 15 min at 40°C, and centrifuged using a B-Braun Sigma 3K2O centrifuge (Sigma-Aldrich Canada, Ltd., Oakville, ON, Canada) 2500g for 5 min. The supernatant was then removed and placed in an appropriately-labeled screw cap centrifuge tube. This extraction was repeated 3 more times on the same flower sample to ensure complete extraction, with the four supernatants pooled in a single centrifuge tube. The volume was topped up to 40mL with the same HPLC grade methanol, at a 98% mixture. This extraction procedure was replicated 2 times on samples representing the pooled capitula of each plant. The final extract was then placed in a -80C freezer until HPLC analysis was carried out.

High pressure liquid chromatography apparatus

For HPLC analysis we used a Gilson System with 811C Dynamic mixer with 306 pump (2) and UV/VIS – 151 Gilson UV detector (Mandel Scientific, Guelph, ON, Canada). The column used was a C18 monomeric column, 300Å pore size, 4.6mm x 250mm (Vydac 238TP series, Hesperia, CA, USA). The chromatographic system was controlled and data analyzed using the Gilson Unipoint Software System (Mandel Scientific, Guelph, ON, Canada).

Chromatographic conditions

The mobile phase used for HPLC analysis was 60/40 acetonitrile and water in an isocratic gradient over 5 minutes with a flow rate of 1mL/min. A volume of 200µl of each sample was injected and read by the UV detector at a wavelength of 260nm, with the column at room temperature.

Method validation

A standard curve of a mixture of the flavonoids quercetin, rutin (Sigma-Aldrich Canada Ltd., Oakville, Ontario), isorhamnetin-3-rutinoside, and isorhamnetin-3-0-glucoside (Indofine Chemical Company Inc., USA), was prepared at concentrations of 0.5, 1.0, 5.0 and 10.0 ppm for each compound. This group of flavonoids elutes together, with one spike, between 2-3 minutes. The flavonoids in the experimental capitula were quantified based on the slope of the standard curve, calculated using known amounts of the mixture of the pure standards. Test samples were also spiked with known concentrations of the standards to obtain the location of each of the peaks for each individual flavonoid and approximate retention time. We were unsuccessful in our attempts to adjust conditions such that they could separate each flavonoid individually;

this is most likely due to the extreme similarity in structure of each of these compounds. Further, specialized analytical methods would be necessary to completely separate these four substances.

Calibration

Standard solutions were prepared at concentrations of 10.0mg/L, 5.0mg/L, 1.0mg/L and 0.5mg/L of each standard in 98% methanol in a volumetric flask. Serial dilutions were created from the stock solution using 98% methanol. A reference standard of the mixture at a concentration of 2.5mg/L was run every 20 experimental samples.

Statistical analyses

Statistical analysis was carried out using SYSTAT version 10.0 (Systat Software Inc., Redmond, CA, USA). Analyses of variance based on the General Linear Model (GLM) were conducted to compare the total flavonoid content of the capitula, followed, where appropriate, by Post-Hoc Sheffé's tests to compare means. Also, nested analyses of variance were performed to determine within-treatment variance of the total flavonoid concentration of the capitula. Tubs with each phosphorus and nutrient supply rate treatment contained both plants that were "grazed", and plants that were ungrazed, which would contribute to within-tub variation.

Results

Table 3.3 shows maximum and minimum values, mean, standard error, and mean/variance ratios for total flavonoid concentrations of the capitula under each

treatment separately and in combination with each other. This table illustrates the high mean-to-variance ratio, which indicates consistent flavonoid concentrations within a treatment.

Table 3.4 summarizes results of the ANOVA in terms of the factors that significantly affect the total flavonoid concentration of the capitula of *C. officinalis* and the capitula flavonoid yield. Phosphorus and nutrient supply rate both had significant effects on the concentration of flavonoids in the capitulum tissues ($p < 0.001$ and $p < 0.01$, respectively). Statisical analysis showed a significant difference in the nested ANOVAs between the replicates within a treatment, for the interaction between P and NSR, as well as in the 3-way interaction between P, NSR and clipping ($p \leq 0.001$ and $p < 0.01$ respectively). Sheffé's post-hoc comparisons of the means were performed to determine which levels of the three treatment variables investigated were significantly different from each other. The results of these comparisons are represented as letters over each bar of the histograms in Fig. 3.3-3.5. Post hoc analyses, comparing mean flavonoid concentrations under contrasting conditions, indicated that by increasing phosphorus concentrations supplied to the plant, the flavonoid concentration per unit dry mass of capitula increases significantly; the highest P concentration (200mg/L) produced the greatest concentration of flavonoids (2.60 mg/g dry mass, Fig. 3.3). The post hoc comparisons also revealed that a more frequent nutrient supply rate (NSR_{24}), where plants received fresh nutrient solution every 24 hours, induced the plant to produce significantly more flavonoids in the capitulum (mean 2.42 mg/g dry mass) than the less frequent nutrient supply rate, NSR_{48}, where nutrient solution was applied every 48 hours (mean 2.32mg/g dry mass, Fig. 3.4). There was no significant effect of clipping on the flavonoid concentration in the capitulum.

Results of the analysis of variance (ANOVA) indicated that the yield (concentration x mass) of flavonoids in the capitula was significantly affected by phosphorus concentration, ($p < 0.001$), clipping, ($p < 0.01$) and the interaction between P concentration and clipping, ($p < 0.01$). A nested ANOVA shows a significant difference between the replicates within a treatment for the interaction of P and NSR, because both "grazed" and ungrazed plant were contained within the same container, and the interactions of the three variables, P, NSR and clipping, $p < 0.001$ for each. Sheffé's post hoc comparison of the means indicates that the two highest P concentrations yielded the greatest total amount of flavonoids per plant with 100mg/L providing the maximum yield (Fig. 3.5a). Post hoc comparison of the effect of the clipping treatment on flavonoid yield shows a significantly higher yield of flavonoids in the treatment that did not experience the clipping treatment (ungrazed) (Fig. 3.5b). The interaction between the variables of P concentration and the clipping treatment occurred at 200mg/L of P with the ungrazed plants generating a greater yield of flavonoids compared to the "grazed" plants (Fig. 3.5c).

Discussion

Flavonoids are found to inhibit or induce enzymes that are involved in mammalian biological pathways that reguate cell division and proliferation, platelet aggregation, detoxification, and the inflammatory and immune response (Della Loggia *et al.*, 1990; Masterová, 1991; Hollman *et al.*, 1996; Karakaya and Nehir EL, 1999). More recently, flavonoids have been considered a most significant anti-carcinogen (Karakaya and Nehir EL, 1999). Yang *et al.* (2000) investigated the chemopreventative effects of diet ingested quercetin and rutin in mice and rats with induced colonic tumorogenesis. The mice and

rats fed the diets with added dietary quercetin and rutin exhibited increased apoptosis of tumor cells in the colon. Ito *et al.* (1999) also showed positive chemopreventive effects of the polyphenols isorhamnetin 3-O-β-D-glucoside and narcissin isolated from *Coleogyne ramosissima* and the polyphenols alienanin B and stenophyllanin A isolated from *Cowania mexicana*. They possess a strong inhibitory effect on Epstein-Barr virus early antigen activation induced by a tumor promoter. They also exhibited anti-tumor promoting activity in two-stage mouse skin carcinogenesis. Several of the polyphenols investigated in these studies are found in *C. officinalis*.

Calendula officinalis contains several phenolic compounds that show potential for human use as an anti-microbial agent. Kalvatchev *et al.* (1997) studied anti-viral effects of *C. officinalis* extracts and found it to be non-toxic to human lymphocytic cells and inhibit HIV-1 reverse transcriptase *in vitro*. In countries around the Mediterranean basin, in Europe and more recently in North America, *C. officinalis* is traditionally used as an anti-inflammatory agent (Masterová *et al.*, 1991; Akihisa *et al.*, 1996; Bezaková *et al.*, 1996); triterpenoids are thought to be responsible for the anti-inflammatory pharmacological effects for which this species is recognized (Della Loggia *et al.*, 1994) as well as flavonoids (Masterová *et al.*, 1991). Recent studies show that *C. officinalis* is also a heart rate inhibitor (Pérez-Gutiérrez *et al.*, 1998), a central nervous system-acting analgesic, causes depression of the cardio-respiratory centres (Rahman *et al.*, 1990), reduces trophic ulcers and their secondary infections (Kartikeyan *et al.*, 1989), shows cytotoxic and anti-tumoral activity (Boucaud-Maitre *et al.*, 1987), and also shows anti-mutagenic activity as a result of saponins in the flowers (Elias *et al.*, 1990). The capitula contain most of the active phytochemicals that are used for medicinal purposes and they are present in capitula in their greatest concentration.

Quercetin, rutin and robinin are the most common flavonoid glycosides in the human diet (Cook and Samman, 1996), obtained most commonly by ingestion of fruits, vegetables, red wine and teas, with a dietary intake of flavonoids in the USA estimated to be in the order of 170mg/day (Cook and Samman, 1996). Quercetin is best studied for its biological effects because it is the predominant flavonoid found in food (Hertog *et al.*, 1993; Karakaya and Nehir EL, 1999). *Calendula officinalis* contains a combination of flavonoids not found in combination in any other plant species analyzed to date (Duke, 2002, Table 3.2). These flavonoids make up approximately 0.28 – 0.85% of the whole inflorescence/dry mass (Piccaglia *et al.*, 1997). A study by Piccaglia *et al.* (1997) found that the two major flavonoids found in *C. officinalis* capitula were a glycoside of quercetin and isorhamnetin-3-O-rutinoside, which were present at concentrations of 1.97mg/g and 1.66mg/g dry mass, respectively, in a given harvest year. Bezaková *et al.* (1996) also confirmed that quercetin and isorhamnetin were the primary flavonoid compounds in *C. officinalis*. Table 3.5 illustrates other concentrations of flavonoids found in various foods.

Flavonoids are universal in angiosperms, gymnosperms, and ferns (Chrispeels and Sadava, 1994), and both quercetin and rutin are quite commonly found in many plants (Duke, 2000), isorhamnetin-3-rutinoside and isorhamnetin-3-O-glucoside are not nearly as common (Duke, 2002). Human use of plant secondary metabolites for their medicinal purposes is a secondary benefit of plant ecophysiology in response to their surroundings. The array of beneficial effects of herbal medicines are often a result of each plant species containing its own, often unique, mixture of secondary metabolites (Freeland and Janzen, 1974), which often act synergistically in a medicinal context by targeting either single, or multiple target sites associated with a physiological process (Briskin, 2000). This

synergy supports the use of whole plant extracts as opposed to the isolation and administration of single compounds. For example, Stermitz *et al.* (2000) demonstrated a synergistic anti-microbial effect of berberine alkaloids with 5′-methoxyhydnocarpin, both extracted from *Berberis fremontii*. It was found that the combined action of these plant extracts effectively inhibited the multi-drug resistance pumps in the human pathogen *Staphylococcus aureus* whereas 5′-methoxyhydnocarpin, alone, did not and berberine, alone, inhibited growth only in much higher concentrations.

Cowan (1999) reviewed a variety of cases where mixtures of plant extracts were shown to have greater antimicrobial and antiviral effects, even greater than isolated, purified compounds. For example, one study reviewed by Cowan (1999) examined activity against Herpes Simplex Virus-1 by isolated flavone and flavonol components. When multiple flavonoids were incubated with the virus simultaneously they were more effective at virus inhibition than the single compounds. A possible explanation of this synergistic effect is that it may be a result of different compounds in the extract weakening numerous defenses in the microorganism.

Results of this study indicate that flavonoid production in capitula can be adjusted either by altering phosphorus concentration or by providing the same amount of nutrients at different rates to the plants. Increasing phosphorus supply significantly increased the production of flavonoids by in terms of their concentration; the highest P concentration (200 mg/L) produced the greatest concentration of flavonoids (mean value 2.60 mg/g dry mass). These results parallel Feller's (1995) finding that increasing the supply of phosphorus for Red Mangrove trees (*Rhizophora mangle*) increased the concentration of phenolics found in the trees. These trees were from a phosphorus-depleted source environment, which is also characteristic of the Mediterranean soil conditions in the

natural range of *C. officinalis* (Cocks and Osman, 1996; Henkin *et al.*, 1996). The carbon/nutrient balance hypothesis, discussed by Bryant *et al.* (1983), Coley *et al.* (1985), Bazzaz *et al.* (1987), and McNaughton (1983) suggests that plants under nutrient limited conditions will allocate more carbon to carbon-based defenses, such as phenolics, when the plant is nutrient-deficient. In contrast, when nutrients are sufficiently supplied, the plant is able to use the carbon for plant structures in vegetative and reproductive growth (McNaughton, 1983). This is because as nutrients other than carbon become limiting in the roots, due to soil deficiencies or ionic competition, their availability to support plant growth is reduced (hence slow-growing species). This, in turn, increases the availability of a pool of nonstructural carbon, available for the production of carbon-based defenses.

The present results do not support the carbon/nutrient balance hypothesis, as increased supplies of P increased the amount of secondary metabolites produced. It is interesting, from a production point of view, to combine the data on concentration of flavonoids with data on the plant tissue analysis presented in Chapter 2. Low P concentrations have the highest concentration of foliar nitrates. If we speculate that higher nitrogen levels in leaf tissue are associated with lower concentrations of secondary metabolites, this would explain the result seen at the lowest concentration of P, where we observe the lowest concentration of total flavonoids, but the highest amount of nitrates in the leaf tissue analysis (Fig. 3.3, 2.15). Furthermore, the amount of P supplied at this low level (10mg/L) may not be sufficient for ionic competition to occur, which allows the relatively greater uptake of nitrogen, therefore decreasing production of flavonoids. The theory that higher leaf nitrogen levels are associated with low secondary metabolite concentration is, then, in agreement with the Carbon/Nutrient Balance hypothesis. An inverse relationship between nitrogen availability and secondary metabolite production

has been illustrated in several studies here; the N:P ratio is critical; it is likely that P is limiting at low P levels; N is limiting at intermediate and high P levels.

Saarinen (1998) investigated the C:N balance by increasing nitrogen concentrations in a common sedge, *Carex rostrata*, grown in a greenhouse. This study also found a shift in the internal C:N balance such that a decrease in the concentration of supplied nitrogen increased the concentration of total nonstructural carbon, in the form of carbohydrates. Mihaliak and Lincoln (1985) found that the concentrations of terpenes, which are also carbon-based metabolites, in *Heterotheca subaxillaris* increased when the availability of nitrogen was decreased. Briskin *et al.* (2000) and Simeunovic (2002) also reported similar results, whereby decreasing nitrogen concentrations in greenhouse-grown *Hypericum perforatum* were associated with increased concentrations of the secondary compound hypericin in leaf tissue.

Nutrient supply rate also significantly affected the concentration of total flavonoids found in the capitula; the more frequent supply rate (NSR_{24}) produced a higher concentration of flavonoids than NSR_{48} (Fig. 3.4). As I showed earlier (Ch. 2), NSR_{24} promoted greater root growth, which is indicative of nutrient limitation/stress. Species like *C. officinalis* have a slow nutrient uptake rate and low capacity for nutrient absorption by the roots. This translates into an excess of nutrient absorption above the level needed to meet growth requirements, during nutrient flushes (Chapin, 1980). It was hypothesized by Clarkson (1967) that frequent supply of nutrients allows luxury consumption, which can lead to toxicity effects in plants. Frequent supplies of nutrient solutions also induce fluctuations in nutrients, pH, and EC. Luxury consumption may also lead to these imbalances causing limitations in uptake of some nutrients even though they are still available in solution (Stadt *et al.*, 1991). Plant tissue analysis showed NSR_{24}

plants contained significantly less leaf tissue nitrogen, in the form of nitrates, than NSR_{48}. These results of the effect of NSR on metabolite production supports the Carbon/Nutrient Balance hypothesis and the theory that limiting nitrogen increases secondary metabolite production, such that the treatment that was limited in nutrients and has lower nitrogen tissue levels had higher total flavonoid concentrations.

In Chapter 2, I showed that the number of whorls of ray florets increased for the nutrient supply rate that provided nutrients less frequently (NSR_{48}). Masterová *et al.* (1991) found that ray florets had a greater total flavonoid content (0.88%) than disc florets (0.25%), by mass. With the production of more ray florets in NSR_{48} we might expect a greater concentration of total flavonoids in this treatment but, on the contrary, plants under NSR_{24} had a greater concentration of flavonoids. This may be attributed to the strength of the effect of detrimental luxury consumption and possible greater fluctuations of nutrients, pH and EC in NSR_{24} that induced flavonoid production to an extent that exceeded the effect of an increased number of whorls of ray florets in NSR_{48}. More whorls of rays indicate more nutrients were available to support structural growth (which is also seen in terms of greater production of other tissues at NSR_{48}), and therefore less nonstructural carbon were available for the production of carbon-based secondary metabolites.

Although there is considerable evidence of an inducible response of secondary metabolite production due to herbivory (Bazazz, 1987; Collantes *et al.*, 1997; Gianoli and Niemeyer, 1997; Agrawal, 2000), the present results do not show a significant increase in flavonoid production per unit dry mass in plants subjected to simulated herbivory as a clipping treatment, compared to ungrazed individuals. It is possible that a greater intensity of grazing may be required to produce a significant difference between "grazed"

and ungrazed plants since only 50% of the leaf tissue was removed from each leaf on one occasion at Day 76 of plant growth. Intensity of grazing is a significant factor for the induction of secondary metabolites (Agrawal, 2000). It is also possible that, while the production of some phenolics, like tannins, are shown to be inducible (Chrispeels and Sadava, 1994) the particular group of flavonoids in *C. officinalis* do not seem to be inducible by simulated herbivore attack at 50% leaf tissue removal. They may be constitutive secondary compounds, meaning the defense is present whether or not a herbivore attacks. Harborne and Williams (2000) noted that it is well established that flavonoids are a constitutive defense as antifungal agents or as phytoalexins. Furthermore Williams *et al.* (2001) investigated the makeup of flavonoids in experimentally grown plants (*Taraxacum officinale)* that were not exposed to grazing and observed significant concentrations of flavonoids in the plants. Our result, in Williams (2000) and in this study, that the secondary metabolites of interest were found within ungrazed plants (constitutively present) is supported by the Carbon/Nutrient hypothesis, in the sense that plants adapted to resource-limited habitats inherently contain higher concentrations of carbon-based defensive compounds (Bryant *et al*, 1983; Coley *et al.*, 1985). In other words, these defensive compounds appear to be regulated by adaptation to the availability of nutrient resources, not induced by the clipping treatment.

With respect to the total yield of flavonoids per plant, as was seen with the concentration of flavonoid per unit dry mass, increasing P concentrations did increase the production of flavonoids. In contrast to the concentration of flavonoid per unit dry mass, the two highest concentrations of P produced the greatest yield. The maximal yield occurred at 100 mg/L of P and not 200 mg/L of P, although these two concentrations were not statistically significantly different from each other. When this result is viewed

in light of results from Chapter 2, for reproductive dry mass and reproductive output, both of which factor into the result of total yield of flavonoids, the two highest P concentrations, once again, did not differ significantly from each other. If we re-examine at these results in terms of the of the amount of tissue nitrates found (Chapter 2), as we did with the results for total flavonoid concentration per unit dry mass, we see the same pattern, with increased tissue nitrogen corresponding to decreased production of secondary metabolites. This result therefore supports Carbon/Nutrient Balance hypothesis discussed above. Simeunovic (2002) also found that increased nitrogen levels decreased the yield of leaf hypericin produced in *Hypericum perforatum* that was grown in greenhouse hydroponics.

There was a significant difference in flavonoid yield per plant with respect to clipping. Ungrazed plants produced a much greater yield of flavonoids than did plants that were "grazed". Results from Chapter 2 also indicated that ungrazed plants had a significantly greater dry mass of capitula than did "grazed" plants. The total yield of flavonoids per plant is driven by the dry mass of the capitula, since yield was calculated using the concentration tissues of the dry mass of capitula. As the mass of the capitula increased so did the yield of flavonoids per plant.

The interaction between P concentration and clipping on the flavonoid yield per plant can be attributed to the individual effects of each variable. The significant difference of the interaction occurs only at the highest P concentration (200 mg/L) with the ungrazed plants showing more flavonoid yield than the "grazed" plants. As discussed previously, the highest P concentration contained the lowest leaf tissue nitrate concentration which corresponds to higher levels of flavonoids. As a result, the effect of

grazing has a more dramatic (and statistically significant) effect at the highest P concentration.

The small, significant difference between the replicates within a tub was an artifact of the experimental design that placed plants that were "grazed" and ungrazed within the same tub for a given P*NSR treatment.

Our findings would support the interpretation of variation in flavonoid concentrations, as we have illustrated in this study with the total flavonoid concentration in the capitula of *C. officinalis,* ranged between 1.006-3.557 mg/g dry mass across all treatments. A principal concern facing the medicinal plant industry is the quality of the product with respect to consistency and uniformity of the concentration of secondary metabolites. There are several cases that illustrate a serious problem of inconsistency of active ingredients in raw plant material and commercially available products. Hepinstall *et al.* (1992) investigated commercial products of Feverfew (*Tanacetum parthenium*) for parthenolide content and found a wide range of parthenolide concentrations, as high as 795μg/capsule, to as low as undetectable levels (<10μg) of parthenolide. The recommended daily dose was one capsule/day for these products, but the parthenolide concentration in them ranged from 795μg/day to less than 10μg/day. Similarly, Feller (1995), found an increase in phenols with increasing phosphorus concentrations in Dwarf Red Mangrove (*Rhizophora mangle*), and Kaundun *et al.* (1998), and reported varying flavonoid concentrations and composition in the same species (*Pinus halepensis*) grown in geographically different locations. Several factors can affect the production of secondary metabolites within a plant population and in different tissues within individual plants. Genetic, as well as environmental and geographic variability have an effect on the flavonoid concentrations produced by a plant (Kaundun *et al.*, 1998). Flavonoids have

been used as markers to identify the floral origin of French honeys, since characteristics of the flavonoid markers found in pollen or nectar are unique to the plant species, and indicate their geographic origin (Soler *et al.*, 1995; Kaundun *et al.*; 1998; Briskin, 2000; Williams *et al.*, 2001). Williams *et al.* (2001) were able to classify 14 species of *Anthemidae* on the basis of their flavonoid profile, in which each species had distinctive constituents. This classification by flavonoids matched that of modern taxonomy. Kaundun *et al.* (1998) also used flavonoid markers to identify the geographical groupings of a single tree species (*Pinus halepensis*) at the infraspecific level, illustrating the influence of environment on the expression of these polyphenols (a group of phenolic compounds) in this species, over genetic determination, yet there are still unique flavonoids markers within the species.

Table 3.3 illustrates that our treatment replicates showed low variance, and high consistency in the total flavonoid concentration within a given treatment, even though the plants used were not genetically identical. This indicates flavonoid concentrations can be controlled by the manipulation of external environmental conditions including resource availability, nutrient concentration and nutrient supply rate. Under greenhouse conditions, external variables can be closely controlled and manipulated, such that the variation in metabolite production between plants may be minimized and a more uniform (and therefore clinically safer) product can be produced.

Conclusion

Increasing phosphorus concentration effectively increased the concentration of flavonoids found in the capitula of *C. officinalis*. This result may not be due to the increase of phosphorus per se, but the indirect effect of a relative reduction in nitrogen

availability caused by the increase in phosphorus. Accordingly, the possible limitation of nutrients due to luxury consumption and fluctuations of nutrients, pH and EC, occurring with the nutrient supply rate that delivers nutrients less frequently also increased total flavonoid concentration. Both of these results support the Carbon/Nutrient Availability hypothesis. Simulated foliar herbivory, performed as clipping 50% of the leaf area, did not increase flavonoid production. This suggests that carbon-based defense compounds, like flavonoids, are not an inducible defense, but are constitutive (Janzen, 1974; Coley *et al.* 1985; Bazzaz *et al.*, 1987) for this type of species adapted to limited nutrient and water resources. Controlled greenhouse hydroponics provided stable growing conditions that, in turn, provide stable concentrations of flavonoids in plants within a treatment. This study showed that greenhouse hydroponics allows the manipulation of variables to increase the output of target secondary metabolites, and that the system provides the control of uniform production of these metabolites, necessary to yield resulting in a consistent, standardized and safer product.

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Table 3.1 Major classes of secondary plant compounds (Adapted from Harborne, 1982)

Class	Subclass
Nitrogen compounds	Alkaloids
	Amines
	Amino acids (nonprotein)
	Cyanogenic glycosides
	Glucosinolates
Terpenoids	Monoterpenes
	Sesquiterpene lactones
	Diterpenoids
	Saponins
	Limonoids
	Cucurbitacins
	Cardenolides
	Carotenoids
Phenolics	Simple phenols
	Flavonoids
	Quinones

Table 3.2. Flavonoids identified in *Calendula officinalis*.

Flavonoid	Author
Quercetin (3, 3′, 4′, 5, 7 pentahydroxyflavone)	Bezakova *et al.*, 1996
Quercetin-3-O-glucoside (Isoquercetin)	Vidal-Ollivier *et al.*, 1989
Quercetin-3-glucorhamnoside	Vidal-Ollivier *et al.*, 1989
Quercetin-2^{G}-rhamnosylrutinoside	Vidal-Ollivier *et al.*, 1989
Rutin (Quercetin-3-rutinoside)	Vidal-Ollivier *et al.*, 1989
Isorhamnetin-3-rutinorhmanoside	Vidal-Ollivier *et al.*, 1989
Isorhamnetin-3-glucorhamnoside	Vidal-Ollivier *et al.*, 1989
Isorhamnetin-3-rutinoside (Narcissin)	Vidal-Ollivier *et al.*, 1989
Isorhamnetin-3-O-glucoside (Isorhamnetin)	Vidal-Ollivier *et al.*, 1989
Isorhamnetin-3-O-α-L-rhamnopyranoayl-(1-2)-O-[α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranose	Masterová *et al.*, 1991
Isorhamnetin-3-O-β-D-glucopyranoside	Masterová *et al.*, 1991
Isorhamnetin-3-O-α-L-rhamnopyranosyl-(1-6)-O-β-D-glucopyranoside (Narcissine)	Masterová *et al.*, 1991

Table 3.3 Maximum and minimum concentrations, mean, standard error of the mean, and the mean/variance ratio of the total flavonoid concentration of the capitula for each treatment.

Treatment	Minimum (mg/L)	Maximum (mg/L)	Mean (mg/L)	Standard Error	Mean /variance ratio
P10	1.006	3.224	2.072	0.060	34.30
P100	1.827	3.557	2.429	0.041	59.27
P200	1.447	3.187	2.594	0.040	65.26
NSR_{48}	1.006	3.557	2.321	0.049	47.72
NSR_{24}	1.827	3.246	2.416	0.039	61.97
U	1.006	3.246	2.341	0.047	49.81
G	1.398	3.557	2.395	0.042	57.27
P10 x NSR_{48}	1.006	3.224	1.989	0.088	22.70
P10 x NSR_{24}	1.427	3.090	2.166	0.080	27.10
P100 x NSR_{48}	1.827	3.557	2.417	0.066	36.65
P100 x NSR_{24}	1.835	3.246	2.440	0.051	47.46
P200 x NSR_{48}	1.447	3.187	2.557	0.064	40.05
P200 x NSR_{24}	2.316	3.314	2.635	0.044	60.03
P10 x U	1.006	3.224	2.007	0.094	21.41
P10 x G	1.398	3.095	2.137	0.076	28.13
P100 x U	1.827	3.246	2.405	0.044	55.27
P100 x G	1.835	3.557	2.456	0.073	33.85
P200 x U	1.447	3.187	2.616	0.070	37.51
P200 x G	1.829	3.128	2.574	0.044	58.98
NSR_{48} x U	1.006	3.224	2.288	0.073	31.29
NSR_{48} x G	1.398	3.557	2.354	0.065	36.46
NSR_{24} x U	1.427	3.246	2.395	0.058	41.27
NSR_{24} x G	1.688	3.128	2.437	0.053	46.38
P10 x NSR_{48} x U	1.006	3.224	1.924	0.142	13.58
P10 x NSR_{48} x G	1.398	3.095	2.055	0.105	19.50
P10 x NSR_{24} x U	1.427	3.090	2.102	0.119	17.63
P10 x NSR_{24} x G	1.688	2.800	2.230	0.108	20.63
P100 x NSR_{48} x U	1.827	2.793	2.394	0.062	38.81
P100 x NSR_{48} x G	1.933	3.557	2.440	0.119	20.55
P100 x NSR_{24} x U	1.929	3.246	2.413	0.062	38.82
P100 x NSR_{24} x G	1.835	3.101	2.473	0.087	28.33
P200 x NSR_{48} x U	1.447	3.187	2.547	0.111	22.94
P200 x NSR_{48} x G	1.829	3.050	2.567	0.067	38.23
P200 x NSR_{24} x U	2.316	3.314	2.707	0.064	42.21
P200 x NSR_{24} x G	2.351	3.128	2.582	0.058	44.52

P10 =Low P (10mg/L), P100 =Intermed. P (100mg/L), P200 =High P (200mg/L), G =Grazed, U =Ungrazed

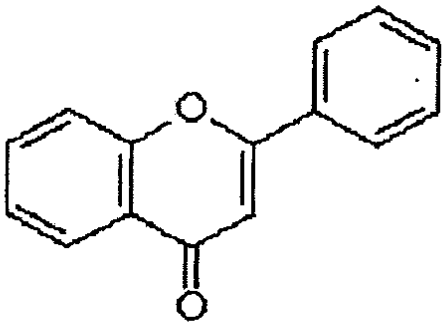
Table 3.4. Overall results of analysis of variance of all factors on total content of flavonoids in the capitula and flavonoid yield.

Source of variation	Flavonoid content per gram dry mass (mg/g)				Total flavonoid content per plant (mg)			
	df	MS	F ratio	P value	df	MS	F ratio	P value
Phosphorus concentration	3	2.9	19.92	p <0.001	2	189.9	17.96	p <0.001
NSR	1	1.1	7.43	p <0.01	1	26.4	2.49	NS
Clipping	1	0.3	1.38	NS	1	95.4	9.02	p <0.01
P*NSR	2	0.4	2.50	NS	2	30.9	2.92	NS
P*clipping	2	0.1	0.43	NS	2	70.6	6.68	p <0.01
NSR*clipping	1	0.006	0.03	NS	1	27.6	2.61	NS
P*NSR*clipping	2	0.05	0.22	NS	2	22.6	2.14	NS
Rep [P*NSR]	24	0.3	2.28	p <0.01	25	58.7	5.55	p <0.001
Clipping*Rep [P*NSR]	24	0.4	2.21	p <0.01	25	42.6	2.46	p <0.001

Table 3.5 Concentrations of quercetin and isorhamnetin-3-O-glucoside (I3OG) found in plants and foods.

Species	Compound	Concentration	Reference
Apple juice	quercetin	0.0012 mg/g	Schieber *et al.*, 2001
Pear fruit (Alexander Lucas)	I3OG	0.0036 mg/g fresh mass	Schieber *et al.*, 2001
Pear fruit (Anjou)	I3OG	0.0009 mg/g	Schieber *et al.*, 2001
Pear (Red Williams)	I3OG	0.0091 mg/g	Schieber *et al.*, 2001
Grapefruit juice	quercetin	0.20-0.88 mg/100ml	Ross *et al.*, 2000
Schisandra chinensis (leaves)	quercetin	1.535-1.140 mg/g dry mass	Sladkovsky *et al.*, 2001
Schinsandra chinensis (caulomas)	quercetin	0.303-0.389 mg/g dry mass	Sladkovsky *et al.*, 2001
Black tea	quercetin	0.0000348 mg/g	Karakaya and Nehir EL, 1999
Linden flower	quercetin	0.0000217 mg/g	Karakaya and Nehir EL, 1999
Sage	quercetin	0.0000272 mg/g	Karakaya and Nehir EL, 1999
Rosehip	quercetin	0.0000167 mg/g	Karakaya and Nehir EL, 1999
Violet carrot juice	quercetin	0.0000837 mg/g	Karakaya and Nehir EL, 1999
Grape molasses	quercetin	0.001692 mg/g	Karakaya and Nehir EL, 1999
Taharra	quercetin	0.0592 mg/g	Karakaya and Nehir EL, 1999
Urtica species	quercetin	0.0087 mg/g	Karakaya and Nehir EL, 1999
Onion	quercetin	3.009 mg/g	Hideaki *et al.*, 1998
Green pepper	quercetin	0.624 mg/g	Hideaki *et al.*, 1998
Fresh hops	quercetin	0.700 mg/g	Peterson and Dwyer, 1998

a) flavonoid skeleton



b) flavonol skeleton

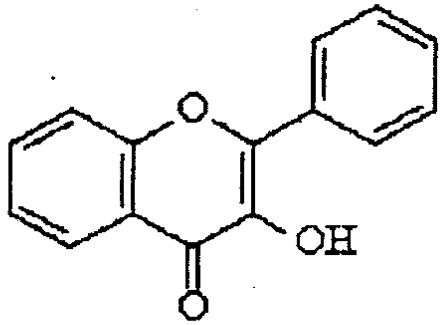


Figure 3.1 General skeleton structure of a) flavonoid and b) flavonol compounds (adapted from Taiz and Zeiger, 1998).

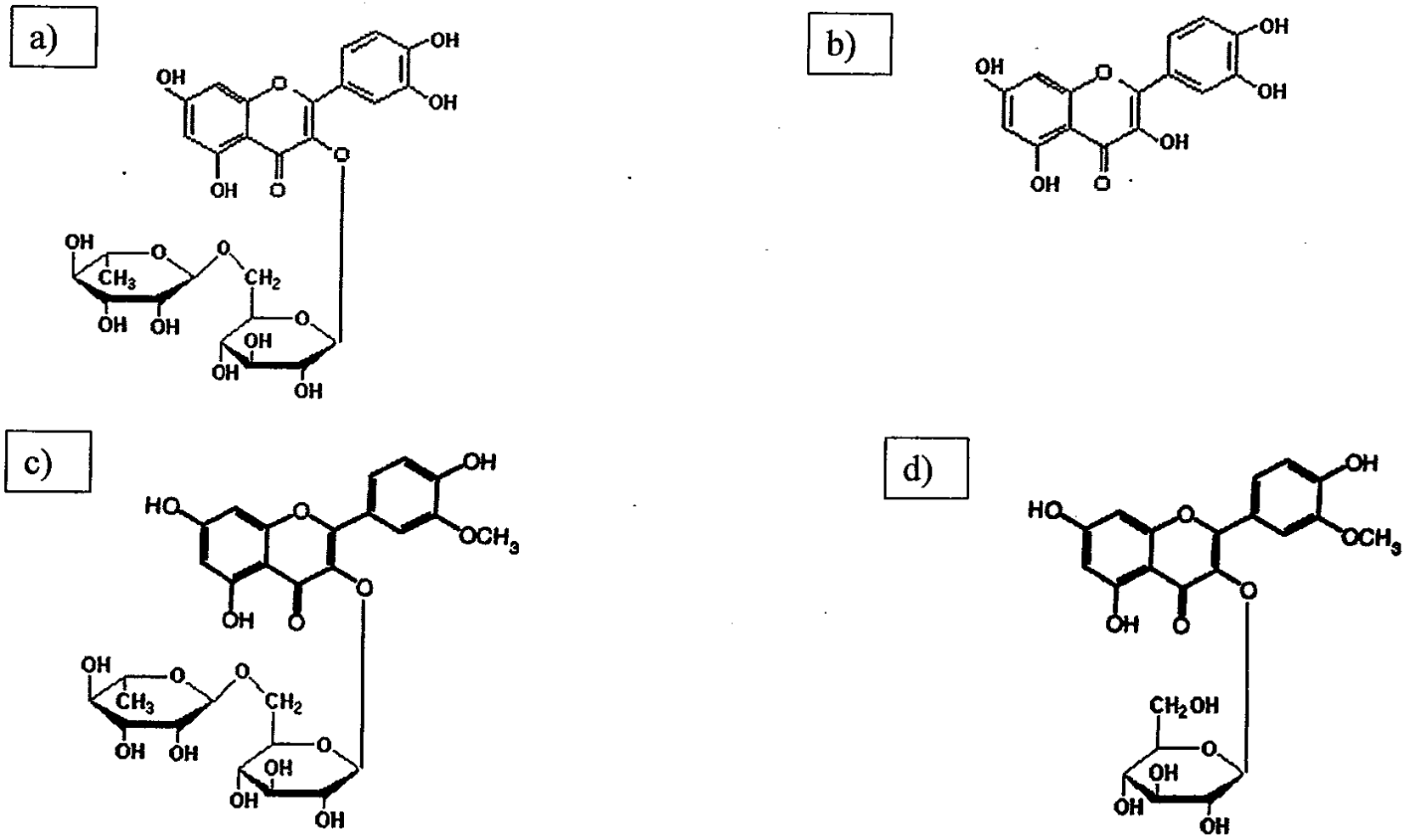


Figure 3.2 a) Structure of rutin molecule (adapted from Boyle, 2000); b) Structure of quercetin molecule (adapted from Paiva, 2000); c) Structure of isorhamnetin-3-rutinoside (narcissin) (adapted from Indofine Chemical Company, 2002); d) Structure of isorhamnetin-3-O-glucoside (adapted from Indofine Chemical Company, 2002).

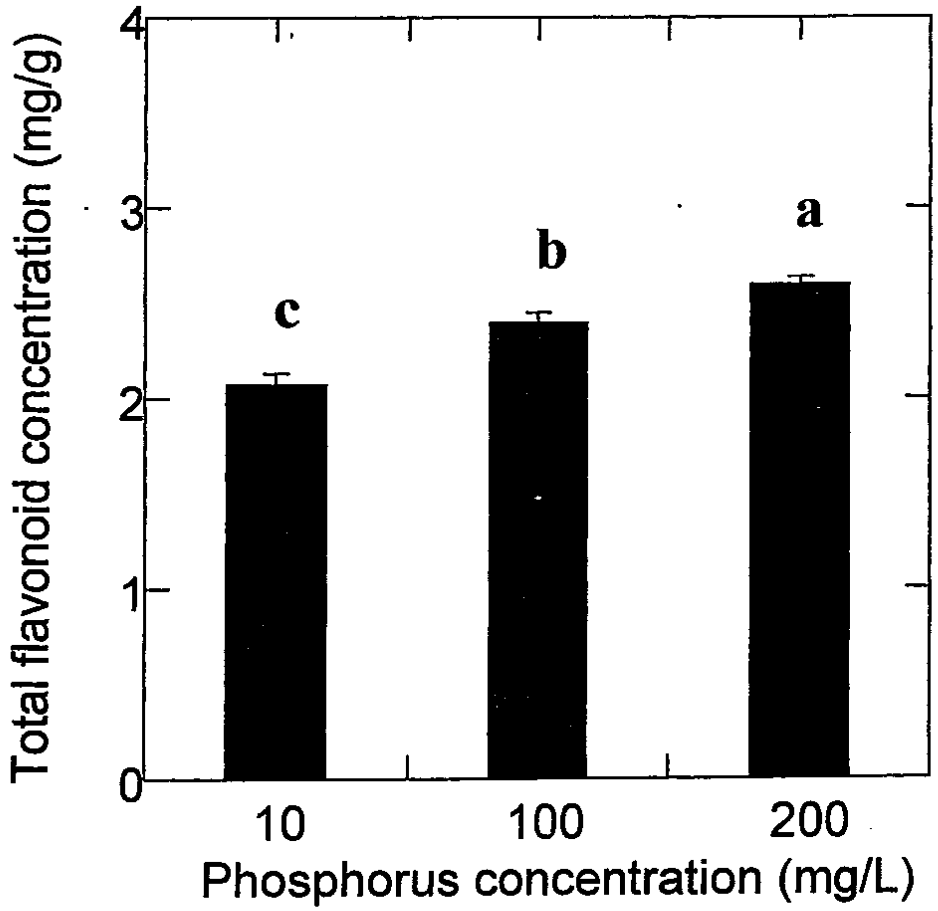


Figure 3.3 Effects of phosphorus concentration on total flavonoid content in capitula. Significant differences in mean values represented by different letters (Sheffé's post hoc comparison).

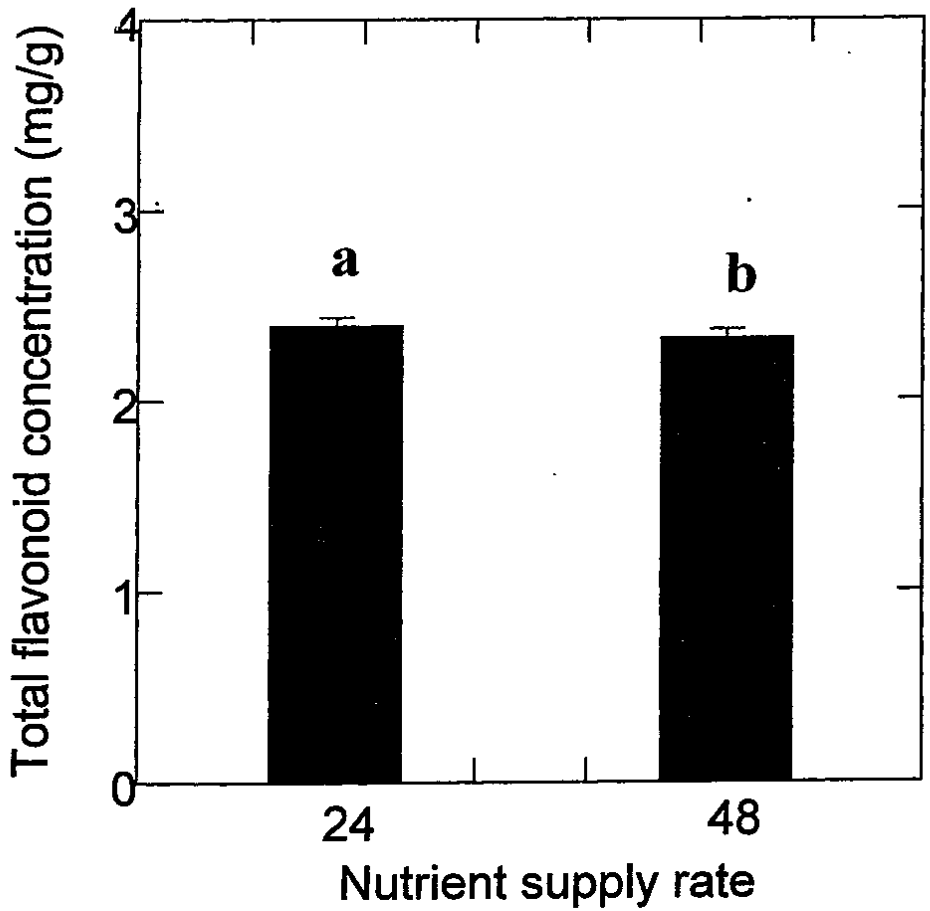


Figure 3.4 Effect of nutrient supply rate on total flavonoid content in capitula. Different letters denote significant differences between mean values (Sheffé's post hoc comparison).

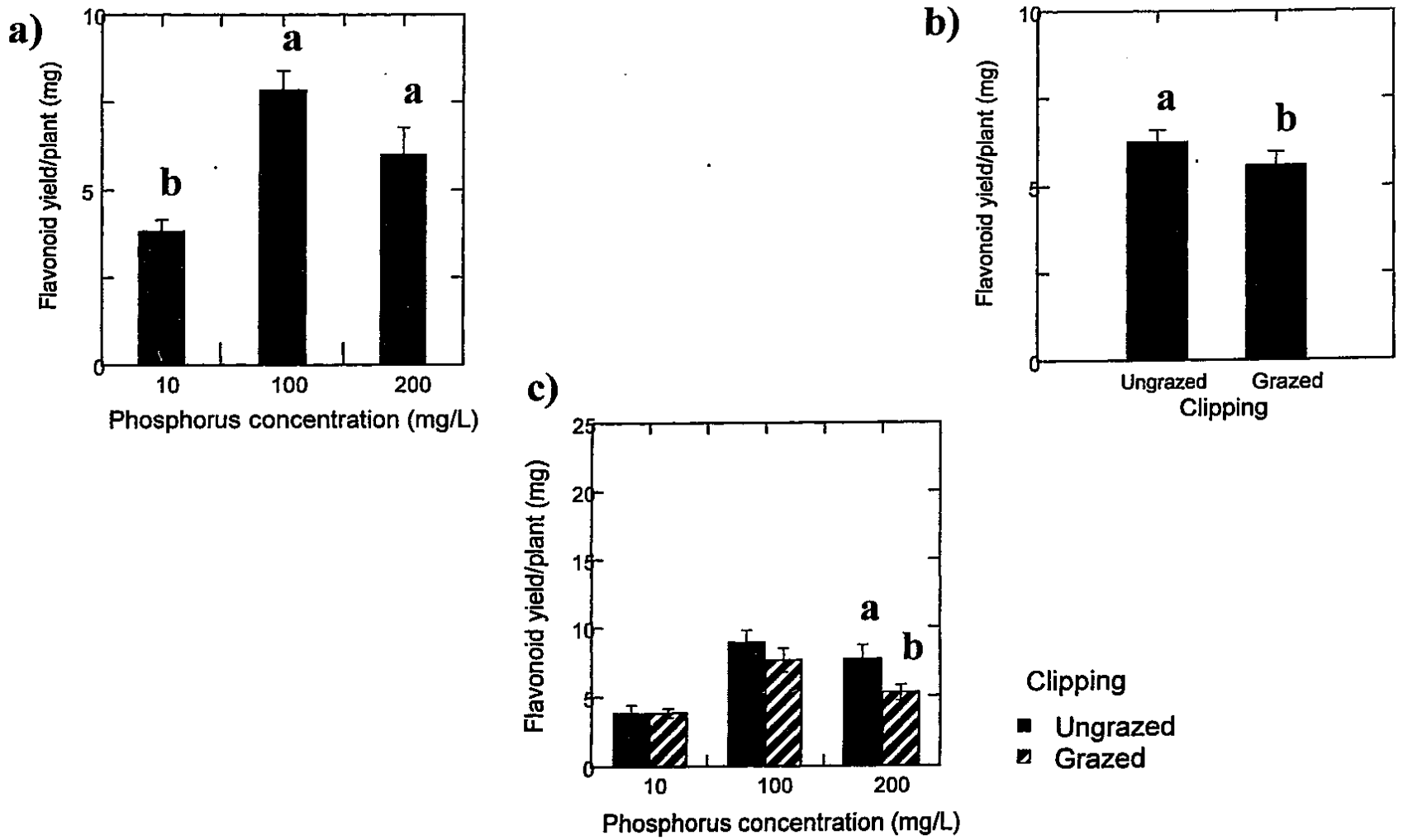


Figure 3.5 a) Effect of phosphorus concentration on total flavonoid content in the whole plant (i.e total capitula); b) Effect of clipping on total flavonoid content in the whole plant (i.e. total capitula); c) Interaction effect of P concentration and clipping on total flavonoid content in the whole plant (i.e. totalcapitula). Differences in mean values represented by different letters (Sheffé's post hoc comparison).

Chapter 4

GENERAL DISCUSSION AND CONCLUSIONS

Nitrogen availability is often the focus of investigations involving nutrients that are limiting to plant growth and reproduction because it is the most limiting nutrient for crop-yields (Gyaneshwar, 2002). Phosphorus follows directly after nitrogen as a major growth-limiting nutrient, even though it can be abundant in some soils, as it is often tied up in organic and inorganic forms not available for plant uptake (Ragothoma, 2000; Rausch and Bucher, 2002). In addition, there is a strong link between the uptake of nitrogen and phosphorus, with emphasis on their ratio to each other (Lapointe, 1985; De Magalhaes *et al.*, 1998). According to the FAO (2000), an estimated 51.6% of the world's soils (3,360,204,000 ha) are phosphorus limited, while only one quarter (24.9%) of the world's soil is nitrogen limited, which are often found together with P-limited soils. Accordingly, many plants have had to adapt to soils with limiting nutrient availability.

In this study, we see that *Calendula officinalis*, which is native to the Mediterranean climate (Duval, 1993) that has phosphorus-limited soils (FAO, 2000), reaches optimal growth of above ground tissue (leaves, stems) in absolute dry mass; and optimal mean dry mass of reproductive tissues at the intermediate level of phosphorus supplied here (100mg/L). Optimal reproductive output, in terms of yield, was seen at 100mg/L and 200mg/L, which were not statistically different from each other. Although, phosphorus may be naturally limiting to this species which grows in P-limited soils, it appears that doubling P availability, from 100mg/L to 200mg/L, does not increase growth and reproduction. This can be explained by an adaptive mechanism for species growing in nutrient limited environments, which is the establishment of a slow rate of growth

(Gerloff, 1963), that decreases the requirement for nutrient uptake, particularly phosphorus (Clarkson, 1967). If a plant has a slow growth rate, nutrients are not needed as frequently for the synthesis of new plant tissues.

With respect to total flavonoid concentration of the capitula and total flavonoid yield per plant, increasing phosphorus increased the production of these secondary compounds. This result is linked to the reduction in relative nitrogen availability, as seen in the nutrient analysis of leaf tissue, upon increasing phosphorus concentrations. The Carbon/Nutrient balance hypothesis states that plants growing in limited-nutrient environments will produce a greater amount of carbon-based secondary plant compounds due to the accumulation of non-structural carbon (Bryant *et al.*, 1983). If soil nutrients are limited, they are not available for the assimilation in structural growth, which causes a build-up of non-structural carbon as carbohydrates, because it is not limited (Ericsson, 1995). The excess carbon is then used to produce the carbon-based defense chemicals, such as phenols (McNaughton, 1983).

Lapointe (1985) found that in addition to the importance of phosphorus availability on the growth of plants, the nutrient supply rate at which it is provided is also critical, even more so than the rate of supply of nitrogen. In addition to the limited P availability of the Mediterranean climate, it is also limited in water availability, due to its xeric moisture regime (Yaalon, 1997). Frequent nutrient/water supply can result in fluctuations in nutrient concentrations, nutrient ratios, electrical conductivity (EC), and pH due to unequal uptake of some nutrients over others or ionic competition (Papadopoulos, 1998). This may result in plant deficiency symptoms and/or fluctuations in plant growth (Stadt *et al.*, 1991). In this study *C. officinalis* responded positively to the nutrient supply rate that provided nutrients more consistently at a less frequent rate, which

provided nutrients/water once every 48 hours. This nutrient supply rate resulted in greater leaf and reproductive dry mass, as well as a greater number of capitula. Slow-growing species adapted to P-limited environments are noted for their luxury consumption of phosphorus at times when phosphorus availability increases, even though it is not needed for growth (Clarkson, 1967; Christie and Moorby, 1975; Chapin, 1980; Lajtha and Klein, 1988). This luxury consumption of P during the more frequent nutrient supply rate, which provided nutrients once every 24 hours, likely resulted in ionic and nutrient imbalances. High P concentrations have been found to lead to P toxicity at 240mg/L (H. A. Mills, 2001, University of Gerogia, Athens, Georgia, pers. comm.), and zinc deficiency (Papadopoulos, 1994).

In contrast, the nutrient supply rate that provided nutrients more frequently, resulted in greater total flavonoid production per capitulum. This is also likely due to the imbalance of nutrients at this supply rate, suggested as a result of luxury P consumption during the nutrient flushes (Stadt *et al.*, 1991), and illustrated by the result of low foliar tissue nitrogen at this nutrient supply rate. As previously stated, the carbon/nutrient balance hypothesis indicates that limited nutrients (nitrogen) increase secondary metabolite production (Bryant *et al.*, 1983), which supports the results seen here.

Plants growing in natural environments are often exposed to grazing by herbivores. As a result of this pressure, plants have adapted with a myriad of responses. Two of these suggested responses, "overcompensation" and increased production of secondary metabolites, were investigated in this study. It was found that a one time simulation of foliar herbivory performed as clipping treatment did not, by itself, induce overcompensation in vegetative or reproductive growth. In fact, control plants significantly differed from "grazed" ones, in that control plants had a greater diameter of

disc florets and greater dry mass of capitula. This suggests that clipping has adjusted the allocation of disc florets, which are hermaphrodite and serve as the male function of the flower. Simulated foliar herbivory results in a decrease of male function/pollen export, which was also found in studies by Lehtila and Strauss (1999) and Freeman and Harper (1980). Overcompensation does not likely occur in this species because of its inherent slow rate of growth; and the tendency for species adapted to nutrient limitation to store excess nutrients during higher nutrient availability, instead of using them for growth enhancement (McNaughton, 1983).

Greater production of flavonoid yield/plant was seen in ungrazed plants compared to "grazed" plants. This result is an artifact of greater dry mass of the capitula in ungrazed plants, since yield was calculated using the values for the capitula dry mass. Therefore, as the capitula dry mass increased so did the yield of flavonoids per plant. Simulated foliar herbivory, performed as clipping 50% of leaf tissue, does not appear to increase the production (inducible defense) of the group of secondary metabolites investigated. These flavonoids were constitutively present within plants that were not "grazed". The Carbon/Nutrient balance hypothesis supports this result, in that it suggests plants adapted to resource-limited habitats inherently contain higher concentrations of carbon-based defensive compounds (Bryant *et al.*, 1983; Coley *et al.*, 1985).

Lastly, this study set out to investigate the possibility of maintaining uniform concentrations of flavonoids within a given treatments, since several studies have shown inconsistencies and variation among metabolite concentrations (Hepinstall *et al.*, 1992; Feller, 1995; Kaundun *et al.*, 1998). It was observed that under closely controlled external variables in greenhouse conditions, variability in metabolite production was

minimized. This control of secondary metabolite production allows for the production of a more uniform product safer for consumption.

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APPENDIX A

Table A.1 General Linear Model results of treatment interactions for P*NSR, P*clipping and NSR*clipping on growth.

Growth Parameter	Source of variation: P*NSR				Source of variation: P*clipping				Source of variation: NSR*clipping			
	df	MS	F ratio	P value	df	MS	F ratio	P value	df	MS	F ratio	P value
Number of leaves	2	81351.4	8.92	p <0.001	2	11348.1	1.24	NS	1	13545.8	1.49	NS
Plant height	2	503.5	4.48	p <0.05	2	269.1	2.40	NS	1	4.5	0.04	NS
Dry mass roots	2	4.9	5.58	p <0.01	2	1.7	1.92	NS	1	0.5	0.54	NS
Dry mass stem	2	6.3	2.82	NS	2	1.1	0.50	NS	1	0.1	0.05	NS
Dry mass leaves	2	13.7	4.91	p <0.01	2	2.6	0.94	NS	1	1.9	0.67	NS
Dry mass buds	2	0.1	4.86	p <0.01	2	0.006	0.61	NS	1	0.000	0.005	NS
Dry mass capitula	2	0.2	0.63	NS	2	0.2	0.86	NS	1	0.003	0.01	NS
Dry mass total reproduction	2	0.5	1.41	NS	2	0.3	0.92	NS	1	0.04	0.13	NS
Total biomass	2	50.9	3.70	p <0.05	2	24.8	1.80	NS	1	4.7	0.34	NS
% Root mass allocation	2	0.001	0.21	NS	2	0.03	5.21	p <0.01	1	0.009	1.70	NS
% Stem mass allocation	2	0.009	3.01	NS	2	0.001	0.22	NS	1	0.002	0.62	NS
% Leaf mass allocation	2	0.004	0.64	NS	2	0.02	3.17	p <0.05	1	0.02	4.22	p <0.05
% Bud mass allocation	2	0.000	1.64	NS	2	0.000	0.65	NS	1	0.000	0.43	NS
% Capitula mass allocation	2	0.002	1.29	NS	2	0.001	0.71	NS	1	0.001	0.45	NS
% Reproductive mass allocation	2	0.003	1.87	NS	2	0.001	1.03	NS	1	0.000	0.23	NS
Root/shoot ratio	2	0.03	1.98	NS	2	0.04	2.16	NS	1	0.004	0.25	NS
Number of buds	2	201.6	5.20	p <0.01	2	8.8	0.23	NS	1	15.4	0.40	NS
Number of capitula	2	92.3	1.68	NS	2	41.0	0.75	NS	1	12.8	0.23	NS
Total reproduction	2	578.5	4.88	p <0.01	2	93.3	0.79	NS	1	80.4	0.68	NS
Average mass/ bud	2	0.000	0.46	NS	2	0.000	0.61	NS	1	0.000	0.83	NS
Av'g mass/capitulum	2	0.001	0.19	NS	2	0.007	1.04	NS	1	0.000	0.006	NS
Average mass/ reproductive structure	2	0.003	2.92	NS	2	0.001	1.01	NS	1	0.000	0.07	NS

Table A.2. General Linear Model results of treatment interactions for P*NSR*clipping, Rep (P*NSR), and clipping*Rep (P*NSR) on growth.

Growth Parameter	Source of variation: P*NSR*clipping				Source of variation: Rep(P*NSR)				Source of variation: Clipping*Rep(P*NSR)			
	df	MS	F ratio	P value	df	MS	F ratio	P value	df	MS	F ratio	P value
Number of leaves	2	2717.6	0.30	NS	30	32764.7	3.59	p <0.001	30	8644.3	0.95	NS
Plant height	2	18.8	0.17	NS	30	816.3	7.27	p <0.001	30	131.9	1.18	NS
Dry mass roots	2	1.0	1.17	NS	30	1.6	1.81	p <0.01	30	0.8	0.79	NS
Dry mass stem	2	6.0	2.66	NS	30	6.0	2.65	p <0.001	30	1.6	0.50	NS
Dry mass leaves	2	4.4	1.58	NS	30	5.3	1.90	p <0.01	30	3.2	0.90	NS
Dry mass buds	2	0.01	1.26	NS	30	0.03	2.41	p <0.001	30	0.007	0.51	NS
Dry mass capitula	2	0.03	0.12	NS	30	1.8	6.54	p <0.001	30	0.3	0.60	NS
Dry mass total reprod'n	2	0.09	0.27	NS	30	2.0	6.06	p <0.001	30	0.4	0.61	NS
Total biomass	2	12.5	0.90	NS	30	32.9	2.39	p <0.01	30	8.5	0.39	NS
% Root mass allocation	2	0.007	1.35	NS	30	0.009	1.81	p <0.05	30	0.009	1.46	NS
% Stem mass allocation	2	0.006	2.11	NS	30	0.003	0.99	NS	30	0.003	0.97	NS
% Leaf mass allocation	2	0.02	3.49	p <0.05	30	0.01	2.16	p <0.01	30	0.01	1.92	p <0.01
% Bud mass allocation	2	0.000	1.05	NS	30	0.000	1.02	NS	30	0.000	0.55	NS
% Capitula mass allocation	2	0.001	1.08	NS	30	0.006	4.94	p <0.001	30	0.002	0.77	NS
%Reproductive mass allocation	2	0.002	1.41	NS	30	0.006	4.36	p <0.001	30	0.002	0.74	NS
Root/shoot ratio	2	0.02	1.28	NS	30	0.03	1.98	p <0.01	30	0.03	1.33	NS
Number of buds	2	0.06	0.002	NS	30	121.3	3.13	p <0.001	30	35.8	0.92	NS
Number of capitula	2	14.2	0.26	NS	30	303.5	5.53	p <0.001	30	64.7	1.18	NS
Total reproduction	2	19.0	0.16	NS	30	669.3	5.65	p <0.001	30	151.5	1.28	NS
Average mass/ bud	2	0.000	0.24	NS	30	0.000	1.19	NS	30	0.000	0.94	NS
Av'g mass/capitulum	2	0.001	0.19	NS	30	0.012	1.76	p <0.05	30	0.004	0.48	NS
Average mass/ reproductive structure	2	0.001	0.72	NS	30	0.002	1.42	NS	30	0.005	0.49	NS

Table A.3. Genderal Linear Model results of interaction of treatments of P*NSR, P*clipping, NSR*clipping on characteristics of capitula.

Capitula characteristics	Source of variation: P*NSR				Source of variation: P*clipping				Source of variation: NSR*clipping			
	df	MS	F ratio	P value	df	MS	F ratio	P. value	df	MS	F ratio	P value
Diameter of whole capitulum	2	0.1	0.16	NS	2	0.2	0.41	NS	1	0.04	0.10	NS
Diameter of disc florets	2	0.2	2.01	NS	2	0.003	0.03	NS	1	0.4	3.42	NS
Number of whorls of ray florets	2	3.7	0.60	NS	2	7.8	1.28	NS	1	2.8	0.45	NS

Table A.4. General Linear Model results of treatment interactions of time and time*P on water relations measurements.

Water Relations Measurements	Source of variation: time				Source of variation: time*P			
	df	MS	F ratio	P value	df	MS	F ratio	P value
Solution volume (before clipping)	12	1965301.1	91.79	p <0.001	24	28671.1	1.339	NS
Solution volume (after clipping)	12	1567073.7	49.84	p <0.001	24	15151.5	0.482	NS
Relative mass of blocks (before clipping)	10	0.1	29.96	p <0.001	20	0.002	0.654	NS
Relative mass of blocks (after clipping)	10	0.1	7.77	p <0.001	20	0.04	4.108	p <0.001

Table A.5. General Linear Model results of treatment interaction of time*NSR, and time*NSR*P on water relations measurements.

Water Relations Measurements	Source of variation: time*NSR				Source of variation: time*NSR*P			
	df	MS	F ratio	P value	df	MS	F ratio	P value
Solution volume (before clipping)	12	1610456.4	75.21	p <0.001	24	9734.0	0.45	NS
Solution volume (after clipping)	12	797980.1	25.38	p <0.001	24	19415.8	0.62	NS
Relative mass of blocks (before clipping)	10	0.01	2.85	p <0.01	20	0.002	0.68	NS
Relative mass of blocks (after clipping)	10	0.4	42.85	p <0.001	20	0.02	2.21	p <0.01

Table A.6. General Linear Model results of treatment interaction effects of P and Rep(P*NSR) on plant tissue analysis.

Nutrient Analyzed	Source of variation: P				Source of variation: Rep(P*NSR)			
	df	MS	F ratio	P value	df	MS	F ratio	P value
Nitrogen	2	4749.7	2.17	NS	29	2113.2	0.97	NS
Phosphorus	2	767.1	1.05	NS	29	820.1	1.13	NS
Potassium	2	1657.0	1.34	NS	29	1296.6	1.05	NS

Table A.7. Mean and standard error (SE) of N, P, and K concentrations in plant tissue analyses.

Variable	P concentration ()		N concentration ()		K concentration ()	
	Mean	SE	Mean	SE	Mean	SE
P10	70.0	7.6	125.5	6.5	246.75	10.7
P100	103.6	11.2	126.5	9.5	238.1	12.2
P200	91.7	7.7	106.7	8.6	223.0	11.9
NSR24	84.2	7.8	112.0	8.8	230.1	9.9
NSR48	91.1	7.3	126.8	4.5	242.0	9.1

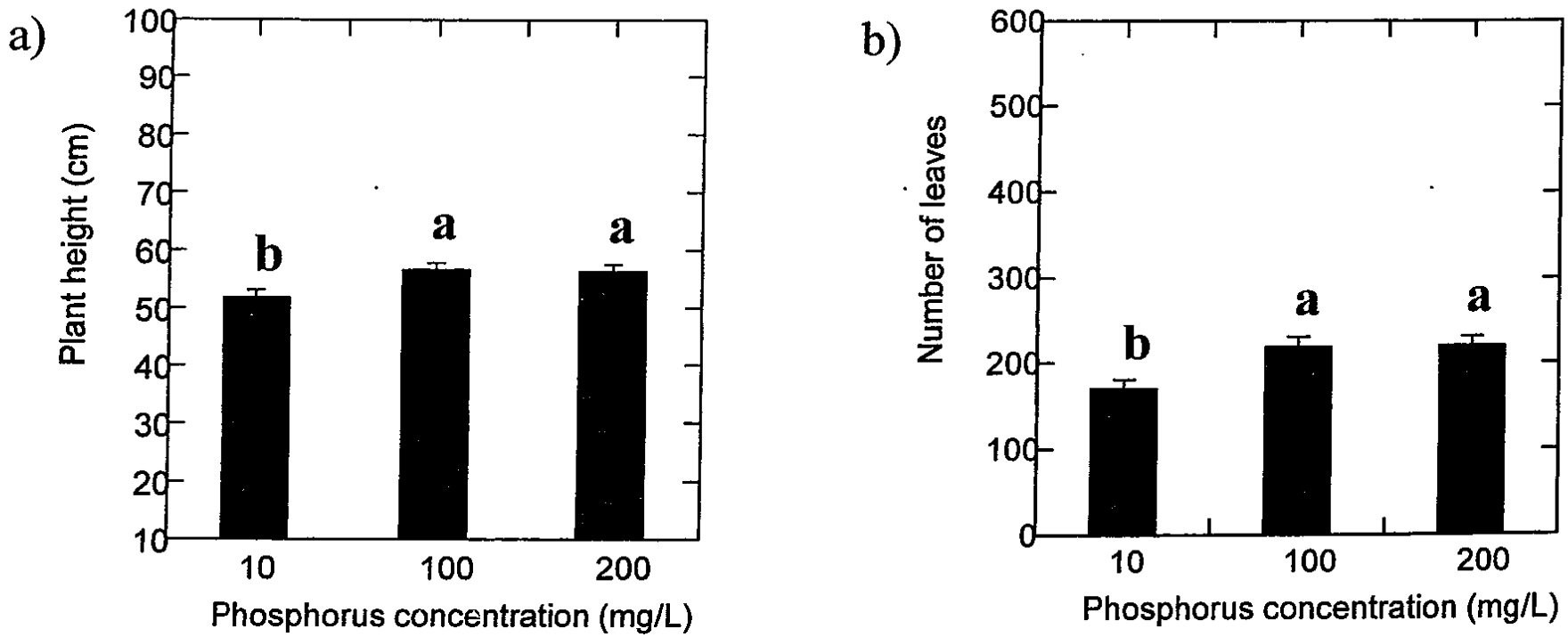

Figure A.1. Main effect of phosphorus treatment on: a) plant height; and b) leaf number. Different letters above a bar indicate significant differences (Sheffé's post hoc conparison).

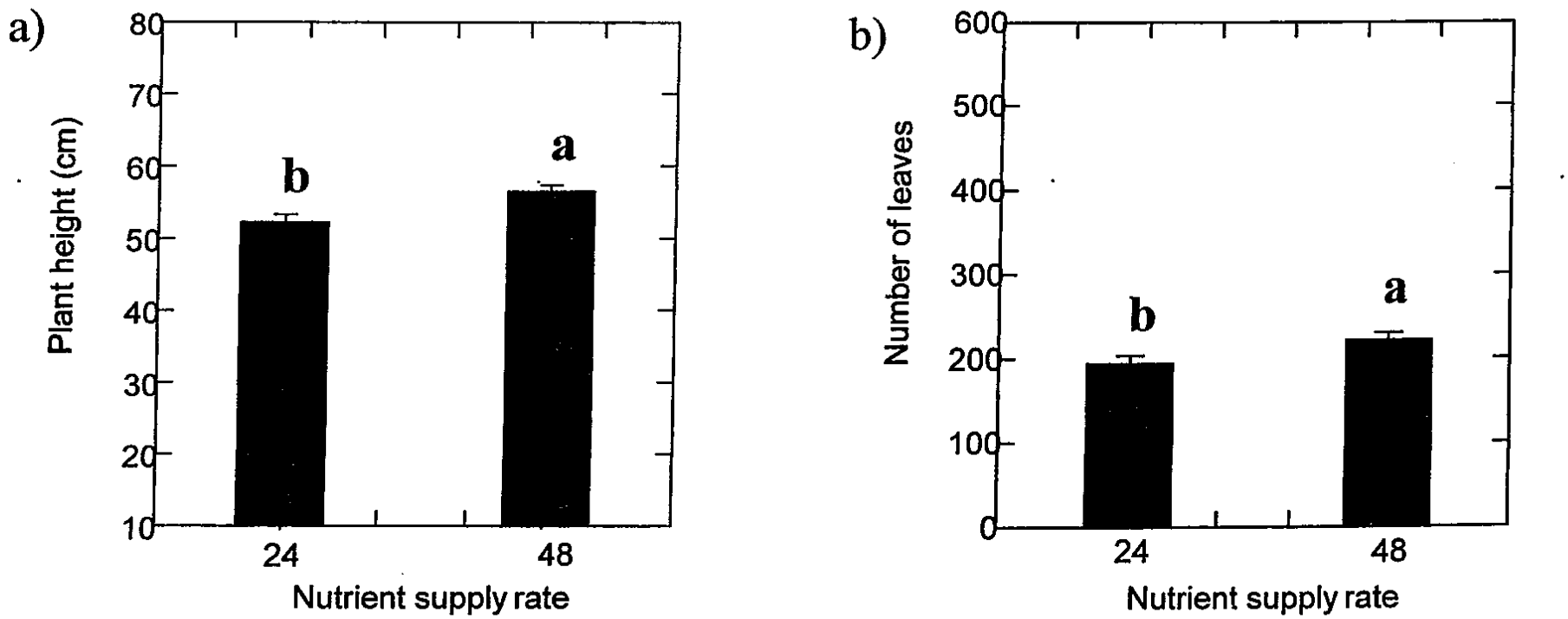

Figure A.2. Main effect of nutrient supply rate on: a) plant height ;and b) leaf number. Different letters above the bars indicate significant differences (Sheffé's post hoc comparison).

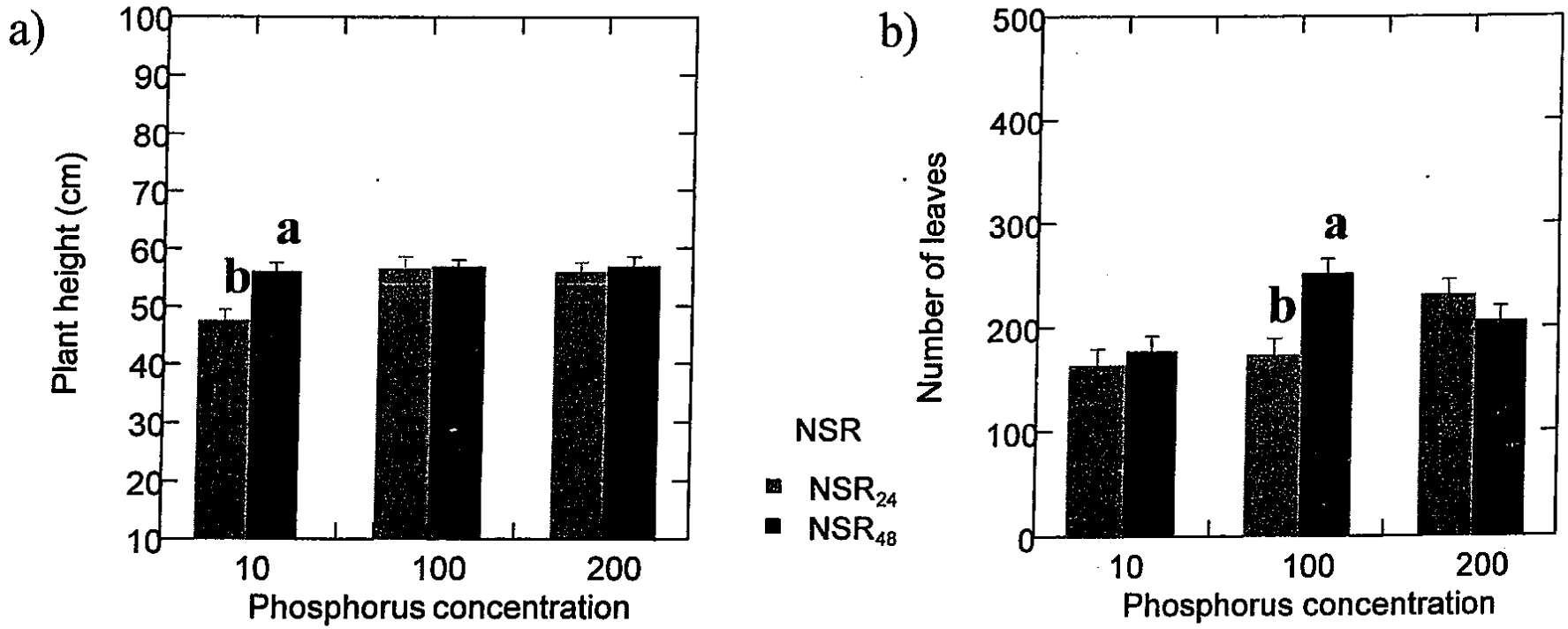

Figure A.3. Interaction of P and nutrient supply rate on: a) plant height; and b) number of leaves. Different letters above the bars indicate where significant differences occur between nutrients supply rates at each P concentration (Sheffé's post hoc comparison).

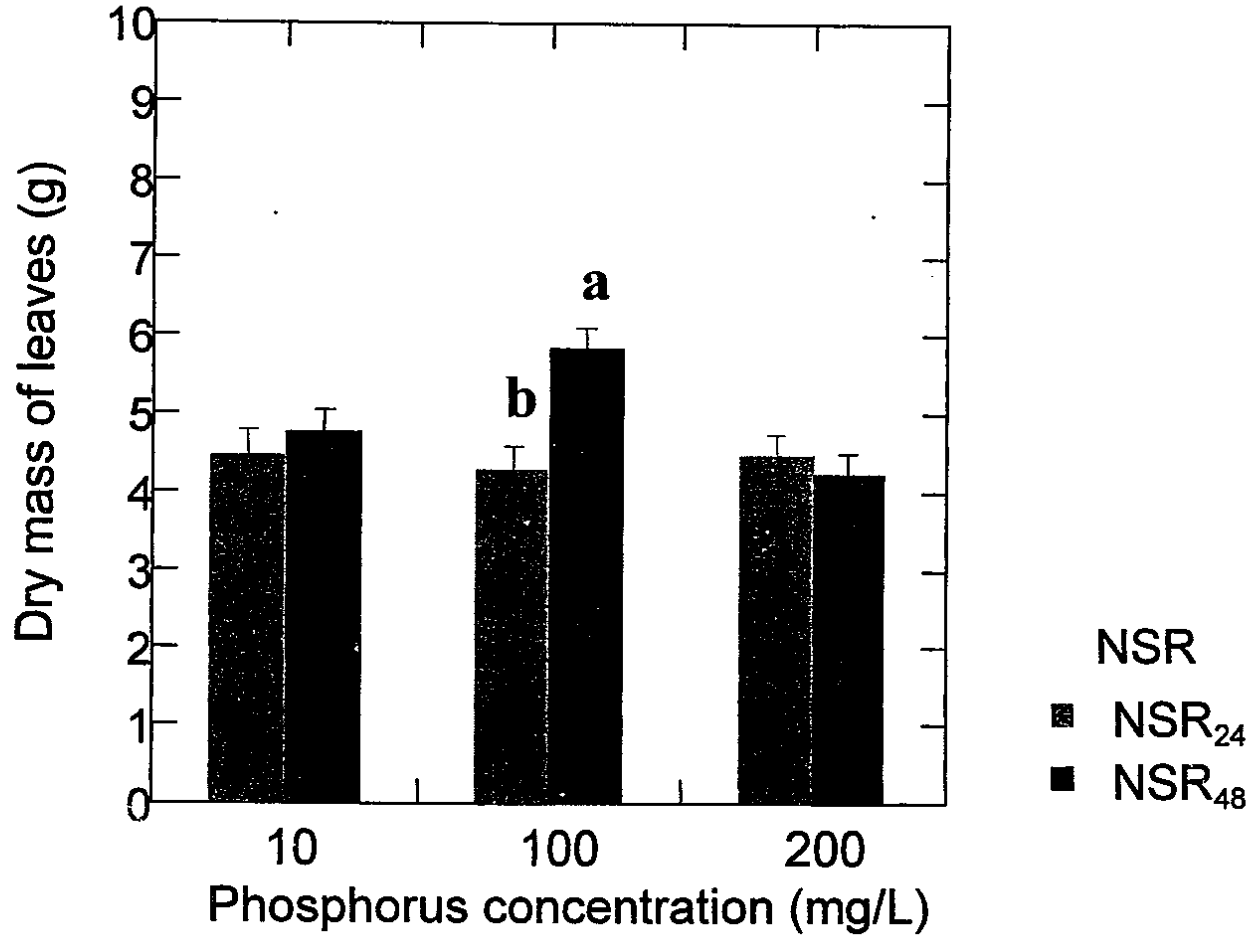

Figure A.4. Interatcion of both P concentration and nutrient supply rate on dry mass of leaves. Different letters indicate significant differences between mean values of the nutrient supply rate at each P concentration (Sheffé's post hoc comparison).

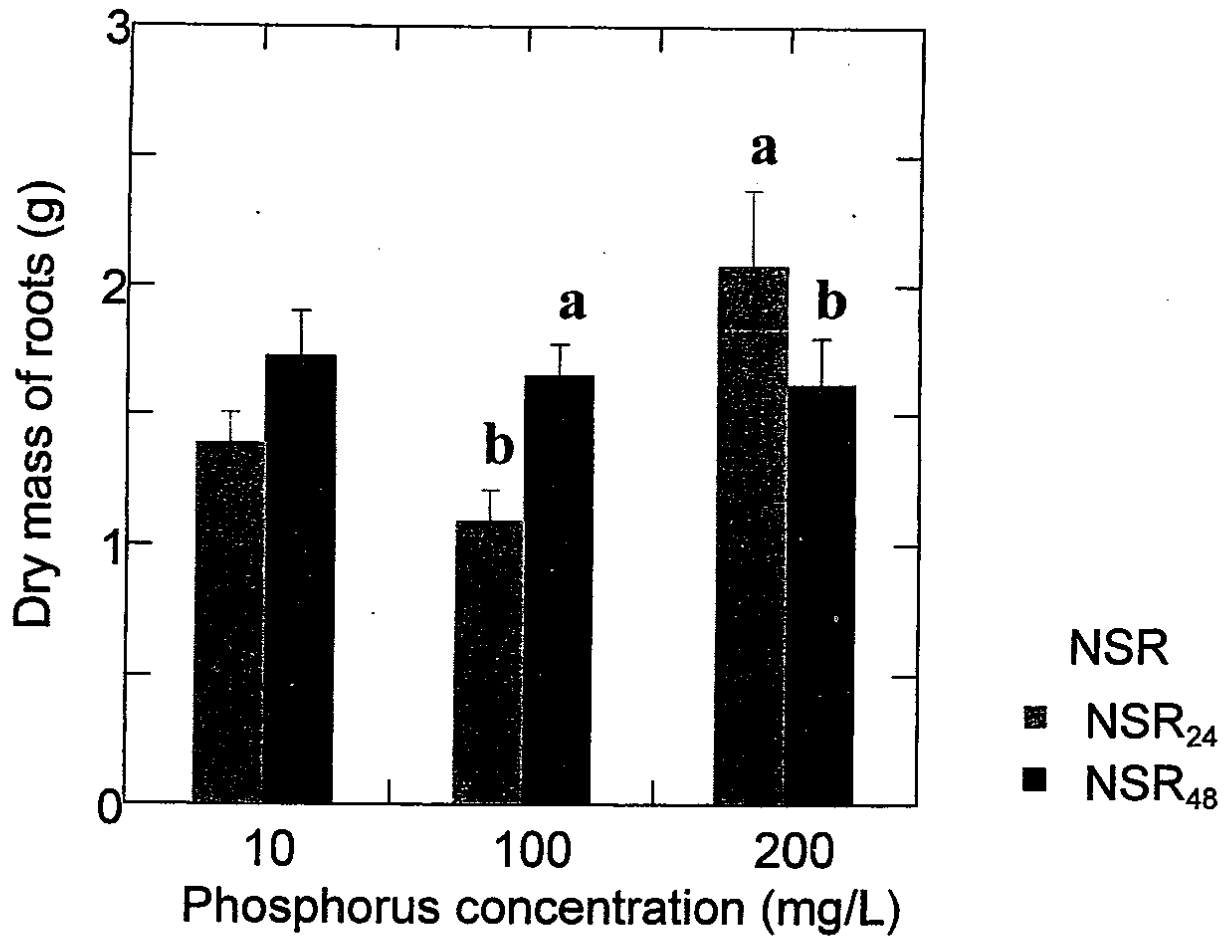

Figure A.5. Interaction of both P concentration and nutrient supply rate on dry mass of roots. Different letters indicate significant differences between mean values of the nutrient supply rate at each P concentration (Sheffé's post hoc comparison).

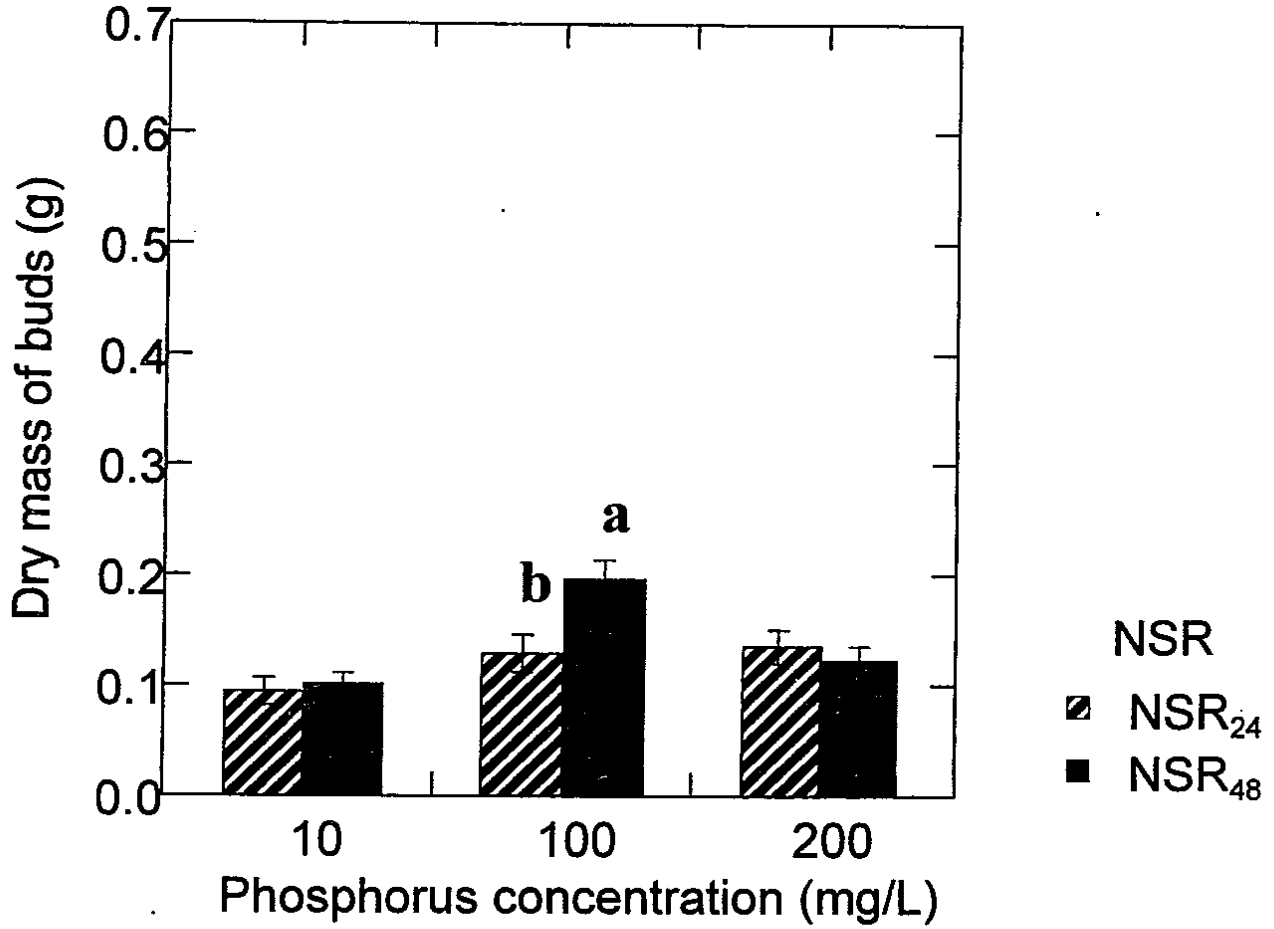

Figure A.6. Interaction of both P concentration and nutrient supply rate on the dry mass of buds. Different letters above bars indicate significant differences between mean values of the nutrient supply rate at each P concentration (Sheffé's post hoc comparison). Where letters do not occur, no significant differences were found.

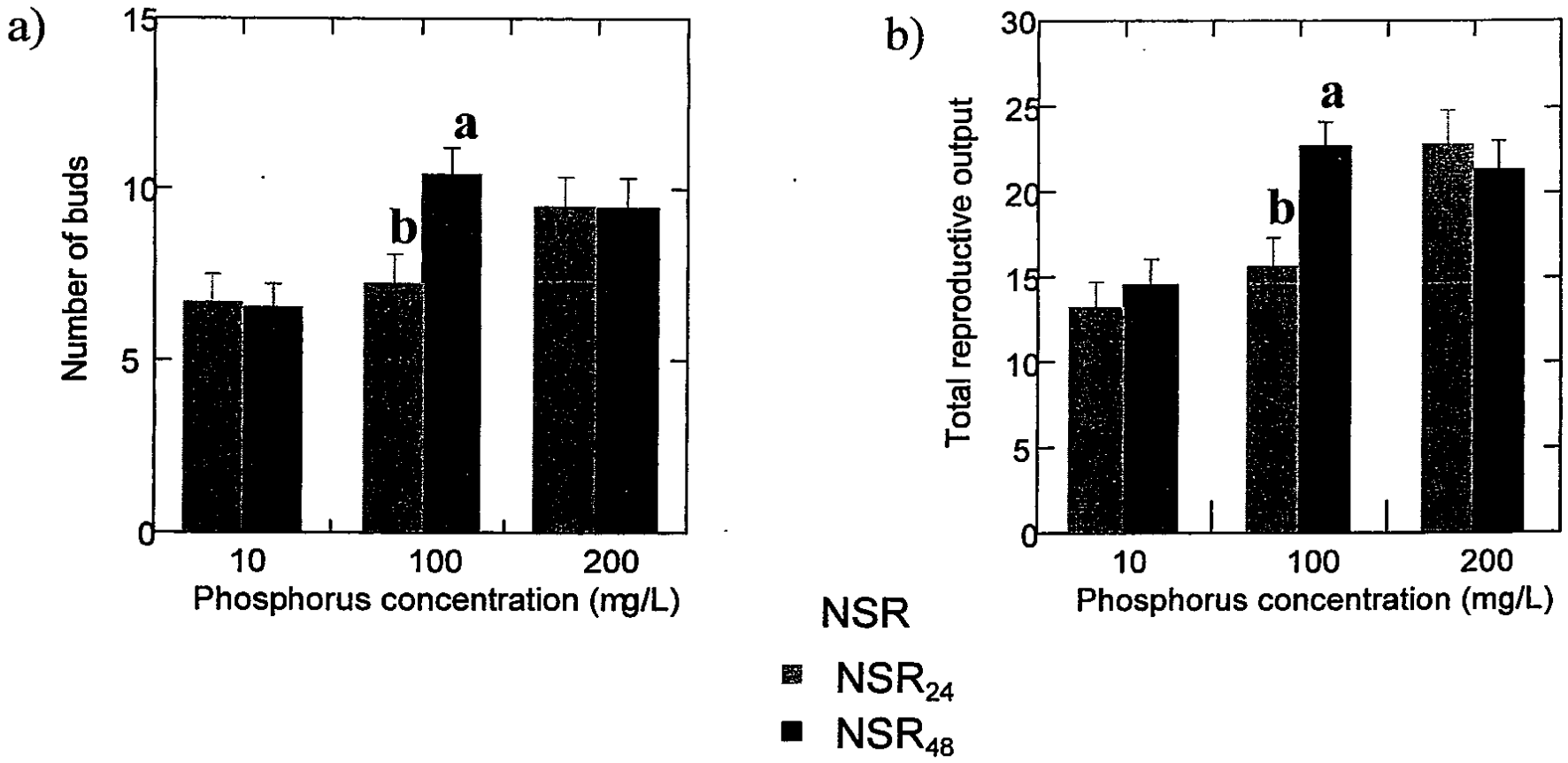

Figure A.7. Interaction effects of P concentration and NSR on: a) bud production; and b) total reproductive output. Different letters indicate significant differences between mean values of the nutrient supply rate at each P concentration (Sheffé's post hoc comparison).

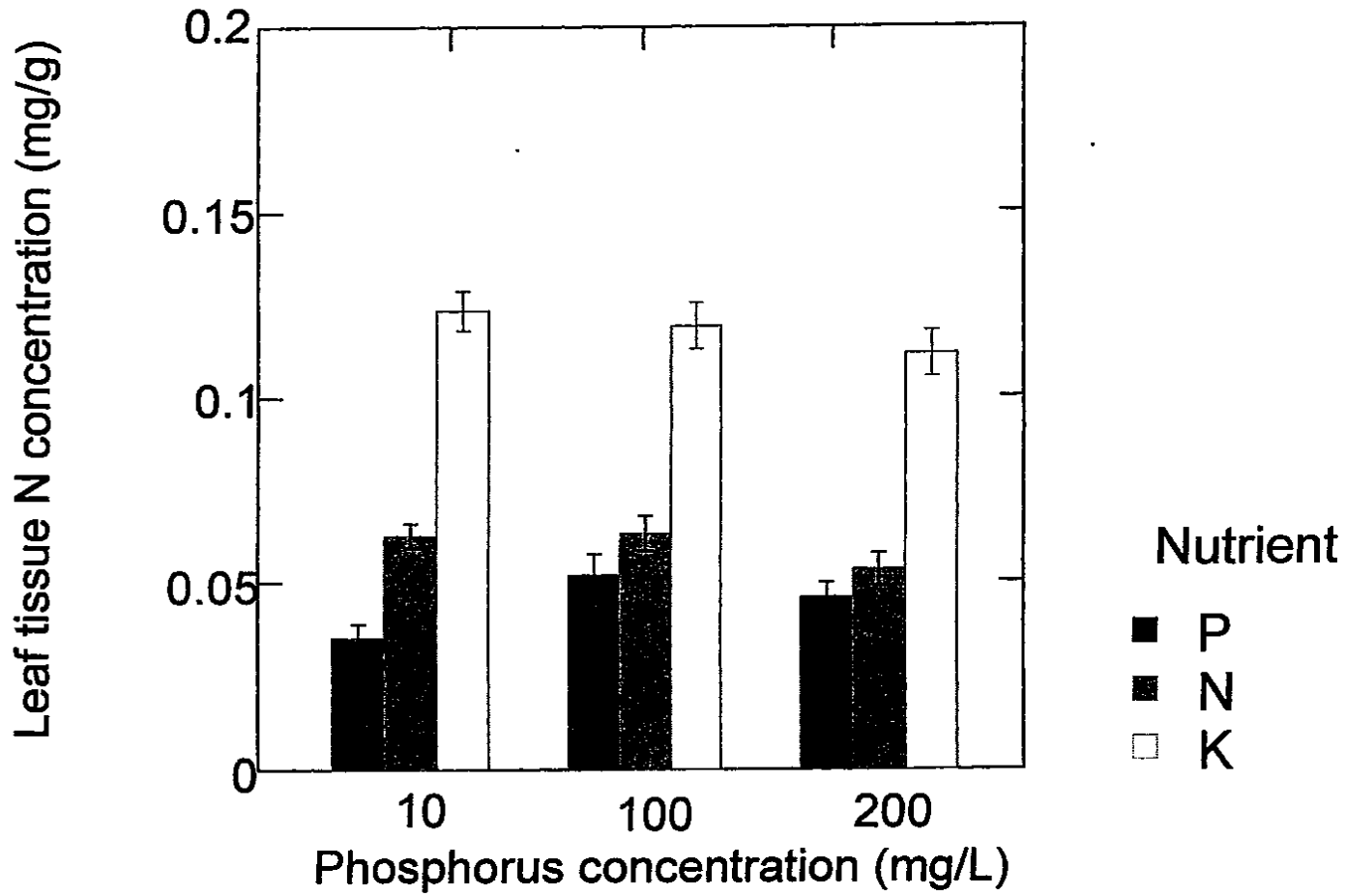

Figure A.8. Trends for tissue concentration of nutrients N, P, K at various P solution concentrations

Plant Tissue Nutrient Analysis

Adapted from Model STH Series Combination Soil Outfit Instruction Manual, LaMotte Company (Chestertown, MD), with the corresponding analysis kit.

Test Methods

Color chart methods are used for all tests except for Potassium. The reaction is performed in a tube or on a spot plate and the resulting color is compared to a laminated color chart. The Potassium test measures the amount of turbidity in a sample relative to the potassium content.

Available Nutrients

All tests measure the portion of the nutrient in the soil that would be "available" for the plant to use.

Extraction Procedure of Green Plant Tissue Tests

Testing an extract prepared from fresh plant tissue provides a means of verifying suspected nutrient deficiencies during plant growth. Plant tissue testing is discussed in the LaMotte Soil Handbook. The necessary information for testing nitrate nitrogen, phosphorus, and potassium in green plant tissues is given below.

Preparation of Tissue Extract

1. Select a small lot of the leaf petioles or succulent portion of the stem. When testing problem plants, collect tissue from those areas where the abnormality is most observable.

2. Use a clean, sharp knife or a razor blade to cut the material into fine bits not more than 1/8 to 1/16" in length and thickness.

3. Weigh out 0.01g of plant tissue and fill an Extraction Tube (0704) with this material. Do not pack down.

4. Add *Universal Extracting Solution (5173PS) to the upper mark. Cap and shake vigorously for five minutes.

5. Use a piece of filter paper (0465) and a plastic funnel (0459), filter the mixture into a second Extraction Tube (0704). This filtrate is the tissue extract to be used in place of the general soil extract in the nitrate nitrogen, phosphorus, and potassium test procedures.

Nitrate Nitrogen

The role of nitrogen in plant nutrition is discussed in the LaMotte Soil Handbook. For interpretation of test results see the LaMotte Soil Handbook and page 22 of the manual.

Procedure:

1. Use a 1 mL pipet (0354) to transfer 1 mL of the general soil extract to one of the larger depressions on a spot plate (0159).

2. Add 10 drops of *Nitrate Reagent #1 (5146).

3. Use a 0.5 g spoon (0698) to add one level measure of *Nitrate Reagent 2 Powder (5147).

4. Stir thoroughly with a clean stirring rod (0519). Allow to stand five minutes for full color development.

5. Match sample color with the Nitrate Nitrogen Color Chart (1315). Record as pounds per acre nitrate nitrogen.

Potassium (Potash)

The role of potassium (potash) in plant nutrition is discussed in the LaMotte Soil Handbook. For interpretation of test results, see the LaMotte Soil Handbook and page 22 of this manual. When present in large amounts, ammonia salts will produce a precipitate similar to that produced by the potassium. It is important that the temperature of the test sample and the *Potassium Reagent C (5162) be in the range of 20-27°C (68-80°F). On warm days, prior to Step 3 below, cool both the test sample in the Potash "A" Tube and the Reagent C contained by placing them in cool water.

Procedure:

1. Use a transfer pipet (0364) to fill a Potash "A" Tube (0245) to the lower mark with the general soil extract.

2. Add one *Potassium Reagent B Tablet (5161). Cap and shake until dissolved.

3. Add *Potassium Reagent C (5162) until the Potash "A" Tube is filled to the upper mark. Allow the *Potassium Reagent C (5162) to run slowly down the side of the tube. Swirl the tube to mix. A precipitate will form if potassium is present.

4. Stand the empty Potash "B" Tube (0246) on the Potassium Reading Plate (1107), a rectangular piece of white plexiglass with a solid black line down the middle. Place the tube directly over the black line.

5. Fill a transfer pipet (0364) with the test sample from the Potash "A" Tube.

6. Slowly add the test sample to the Potash "B" Tube, allowing it to run down the side of the tube. Observe the black line down through the Potash "B" Tube. Continue to add the test sample until the black line just disappears.

7. Record as pounds per acre Available Potassium where the level of the liquid meets the scale printed on the side of the Potash "B" Tube.

8. If the test result is equal to or greater than 400 pounds per acre, repeat the test on a diluted test sample as follows:

A. Fill a Potash "C" Tube (0247) to the lower mark with the general soil extract.

B. Add *Universal Extracting Solution (5173) to the upper mark and mix.

C. Using this diluted extract follow Steps 1 through 7 above. Multiply the test result by 2 to obtain pounds per acre Available Potassium.

Phosphorous

The role of phosphorous in plant nutrition is discussed in the LaMotte Soil Handbook. For interpretation of test results see the LaMotte Soil Handbook and page 22 of the manual. The Phosphorous test is extremely sensitive. Special precautions should be taken to prevent contamination. In particular, exposure of the test components to fertilizer dust must be scrupulously avoided. The operator's hands and clothing, the work surface, and the testing area in general must be clean and free of fertilizer residues.

Procedure:

1. Use a transfer pipet (0364) to fill a "Phosphorous B" Tube (0244) to the mark with the general soil extract.

2. Add 6 drops of *Phosphorous Reagent 2 (5156). Cap and shake to mix.

3. Add one *Phosphorous Reagent 3 Tablet (5157). Cap and shake until dissolved.

4. Immediately compare the color that develops in the test tube against the Phosphorous Color Chart (1312). Hold the tube about one inch in front of the white surface in the center of the color chart. View the chart and sample under natural light for optimum color comparison. The test result is read in pounds per acre Available Phosphorous.

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